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TITLE: Molecular Pathogenesis of Rickettsioses and Development of Novel
Anti-Rickettsial Treatment by Combinatorial Peptide-Based Libraries

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14. ABSTRACT The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsiae and to study the molecular pathogenesis of rickettsioses. The following specific aims were proposed: 1) To establish heterogeneous cell populations, with each cell expressing a unique member of a complex combinatorial peptide-based (e.g., adaptein) library and challenge with <i>R. prowazekii</i> , <i>R. rickettsii</i> , and <i>O. tsutsugamushi</i> ; 2) To determine the role of NF-κB, cytokines, ROS and NO in intracellular killing of rickettsia-infected monolayers containing adapteins and 3) To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability. Work on specific aim 1 was partially successful. Resistant colonies of 20-25 cells were obtained after rickettsial challenges. However, expansion of such colonies was not possible. Great progress was made on specific aims 2 and 3. The role of rickettsiae, cytokines (IFN-γ, TNF-α, and IL-1β), ROS and NO in endothelial permeability was very well characterized in vitro. Changes in occludin, p120 and β-catenin have also been documented by confocal microscopy and are related to increased endothelial permeability. mRNA microarray experiments revealed differences between infected and non-infected endothelial monolayers and between <i>R. conorii</i> and <i>R. rickettsii</i> -infected endothelial monolayers.					
15. SUBJECT TERMS <i>Rickettsia rickettsii</i> , <i>R. prowazekii</i> , <i>R. conorii</i> , adapteins, microvascular permeability, epidemic typhus, rocky mountain spotted fever.					
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Table of Contents

Introduction.....	5
Body.....	6
Key Research Accomplishments.....	20
Reportable Outcomes.....	22
Conclusion.....	25
Scientific personnel.....	27
References.....	29
Appendices.....	32

I. INTRODUCTION

Rickettsiae are obligately intracellular organisms that have evolved in close association with an arthropod host. Diseases caused by these organisms are still prevalent in many parts of the world and include Rocky Mountain spotted fever (the most common rickettsiosis in the US), epidemic and endemic typhus (1-4). The latter two are still responsible for thousands of deaths around the world every year.

Rickettsia prowazekii and *R. rickettsii* are listed in the Select Agents Act and are part of the Centers for Disease Control and Prevention (CDC) and NIH category B agents and the North Atlantic Treaty Organization (NATO) select agent list for their potential use as bioterrorist/biowarfare agents (5-8). The most feared complications of rickettsial infections are the development of severe cerebral and pulmonary edema leading to permanent neurologic sequelae or death owing to respiratory failure (3,4).

The target cell of rickettsial pathogens is the endothelium lining the vessels of the microvasculature, as demonstrated by studies performed on autopsy cases dying of rickettsioses and animal models (9,10). The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens. In addition, molecular pathogenesis of rickettsioses is being studied by developing *in vitro* models to study endothelial permeability and intracellular rickettsial killing in both wild type and adaptein protected cells. The long term objective of this proposal is to develop new treatments for rickettsioses and to identify novel molecular targets of rickettsial pathogenesis that would provide sites for new therapeutic interventions, and to eventually use these targets to develop effective and rapid BWT countermeasures. The new therapeutic interventions are

justified due to the narrow range of antimicrobial agents available for rickettsiae and *Orientia*, emergence of chloramphenicol- and tetracycline-resistant strains of *Orientia*, and the possibility of genetically engineered resistance.

II. BODY

Specific aim # 1: To utilize retrovirally encoded adapteins to generate cell monolayers resistant to rickettsial challenge.

During the first year of the project we were able to construct two dozen libraries using plasmid p_{tat}CCDlib- β -gal encoding combinatorial 6-mer, 12-mer, and 18-mer with diversities of $\sim 10^7$ - 10^8 peptides (11,12). The combinatorial libraries were successfully produced in large scale using bacterial cells. An additional library was constructed using an EGFP-based scaffold for library presentation. The presence of the EGFP scaffold allowed us to track retrovirally infected cells and survivors after rickettsial challenges more efficiently. Initial experiments for rickettsial challenges were performed with Vero cells and SVEC cells (murine lymph node endothelial cells). 90-100% of cells were infected with the retrovirus-containing adaptein libraries. Several challenging experiments were performed by using different rickettsial MOI. Experiments were not successful due to the type of cell monolayers used which made it difficult to select resistant clones. During the second year of the contract we concentrated on using the enhanced libraries containing the EGFP scaffold and 6-mer, 12-mer and 18-mer adapteins with diversities between 2×10^7 to $3-5 \times 10^8$ (13). We also decided to use a different cell line due to the difficulties we had with Vero cells and SVEC. A rat-derived brain endothelial cell (RBE4) was optimized for use. Infection with the retroviruses containing adapteins was 90-100%.

Cells were infected at MOI of 25 with highly purified rickettsiae and re-challenges were performed 2-3 days later with 15 MOI. Three additional rechallenges with MOI of 10 were also performed. Several foci of possibly resistant clones of RBE4 cells were detected after five challenges. However, the surviving cells were expressing EGFP suggesting that the retrovirus was not present in such cells. In addition, further characterization of the clones was impossible due to inability of the cells to multiply. Several other challenge experiments were conducted under similar conditions and “resistant” clones expressing EGFP were obtained. Characterization of the clones was again impossible due to inability to expand the resistant colonies containing 20-25 EGFP expressing cells. Several methods were used trying to expand these “colonies” including enrichment of medium, addition of non-transformed RBE4 in order to increase quorum sensing, and use of small culture vessels. During this second year challenging experiments were performed with *R. prowazekii*, *R. rickettsii* and *O. tsutsugamushi*, all yielding similar results (13). During the third year of the contract our laboratory acquired two human-derived endothelial cells lines from brain and skin, namely SV-HCEC and TIME (SV40-transformed human microvascular endothelial cells and telomerase-immortalized microvascular endothelial cells, respectively). Both cell lines were infected with the pantropic retroviruses containing 6-, 12-, and 18-mer adapteins. Cells were initially challenged with 25 MOI of *R. rickettsii*, *R. prowazekii* and *O. tsutsugamushi*. Three to four rechallenges were performed with 10 MOI and resistant colonies were again observed, expressing EGFP and containing 10-20 cells. Expansion of the colonies was again impossible under several different conditions (14). During the 4th year of the project we decided to try

cell suspensions instead of cell monolayers. The rationale behind this approach was the use flow cytometry/cell sorting so that resistant cell populations expressing EGFP could be sorted using EGFP expression and propidium iodide exclusion. For these experiments we used a human monocytic leukemia cell line (THP-1) treated with vitamin D3, and a murine monocytic cell line treated with dexamethasone to induce differentiation down the macrophage lineage. Both cell lines were infected with the pantropic retroviruses efficiently. Challenge and rechallenge experiments were performed using the same MOI's used for the cell monolayers. Cells surviving the challenges were sorted out successfully but we had the same difficulties as before: expansion of the resistant clones was not possible. It is likely that the retroviral infection of all cell lines used in the challenging experiments affects the capacity of the cells to multiply profoundly. We still continue to perform experiments with the hope that resistant clones will be able to multiply in culture medium by changing the growth environment of the cells. Alternatively, we are using THP-1 and M1 cells without treatment with vitamin D3 or dexamethasone. A more primitive phenotype could favor cell multiplication.

Specific aims #2 and #3: To determine the roles of NF- κ B, cytokines, ROS and NO in intracellular killing of rickettsia-resistant monolayers. To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability seen in rickettsial infections

NF- κ B activation

During the second year of the contract experiments to demonstrate NF- κ B activation were performed using RBE4 cells. They were seeded in 24-well plates and

transfected at semiconfluency with pNF- κ B-d2EGFP vector plasmid (Clontech) by using LipofectAMINE PLUS reagent. Optimization of the transfection protocol was performed according to manufacturer's instructions by using different concentrations of plasmid DNA (range: 1.0-1.6 μ g). The transiently transfected cells were infected with *R. rickettsii* at 10 MOI and were then monitored under an inverted Olympus microscope equipped with UV light and EGFP filters. Cells were evaluated every 30 minutes for 2 hours. NF- κ B activation was demonstrated in infected cells as emission of green fluorescence. Negative controls included in the experiment included non-transfected and non-infected RBE4 cells (13).

Reactive Oxygen Species

The role of ROS in rickettsial killing was demonstrated by the use of sulfoximine in order to decrease intracellular glutathione levels (12). We treated cell monolayers for 24 hours with sulfoximine previous to rickettsial inoculation and sulfoximine-treated and non-treated cell monolayers were then infected with 10 MOI of *R. rickettsii*.

Based on propidium iodide staining, cell death was increased 50-100% when glutathione levels were depleted suggesting a role for ROS in intracellular killing of rickettsiae. We also performed several experiments to elucidate the role of ROS in increased vascular permeability across endothelial cell monolayers. In order to do so, 10 U/ml of catalase, 1 mM of N-acetyl-cysteine, and α -tocopherol at a concentration of 30 μ M were added to rickettsia-infected monolayers. All three compounds were added both before, and at the time of infection of the monolayers. No differences were observed between infected or non-infected monolayers when catalase (ROS scavenger) or N-acetyl-cysteine (glutathione replenisher) was added to the medium.

However, pre-treatment of the monolayers with α -tocopherol (anti-oxidant, ROS scavenger) prevented a 5-10% increase in permeability as compared to infected monolayers that were not pre-treated with α -tocopherol (14,15). Cell survival in the monolayer was marginally improved as shown by loss of micromotion in the non-treated monolayers 2 hours earlier when compared to the treated monolayers (14).

Nitric Oxide

The role of nitric oxide in rickettsia-infected endothelial cells as a rickettsicidal agent and as a mediator of increased permeability across cell monolayers was also studied using human microvascular endothelial cells. We first studied the effect of exogenous nitric oxide on rickettsial gene copy numbers using a nitric oxide donor (DETA NONOate) (14,16-19). Endothelial cells infected with *R. conorii* were treated with 100 μ M and 500 μ M of DETA NONOate and copy numbers of rickettsiae were determined at 24, 48 and 72 hours. No significant changes were observed at 24 or 48 hours in rickettsial copy numbers in infected and non-infected cells. However, at 72 hours, we detected 23 times as many rickettsiae in non-treated cells than cells treated with either 100 or 500 μ M of DETA NONOate. The effect of DETA NONOate in endothelial permeability was also studied. At 100 μ M, we saw no statistically significant increase in endothelial permeability over 4 days of experiments but at 500 μ M a marked increase in permeability was observed after 24 hours (17).

These studies have revealed that NO is very effective at limiting the number of intracellular rickettsiae. Likewise, we have shown that certain lower levels of NO-donors can effectively extend the life-span of endothelial monolayers and have relatively little effect on the barrier function of the monolayer. Conversely higher

levels of the NO-donor produce dramatic changes in the integrity of the monolayer by first stabilizing the monolayer and then causing a slow and steady loss of barrier function. Additionally we have seen an abrogation of rickettsia-induced endothelial permeability in the presence of the broad-spectrum NOS inhibitor L-NAME (16). In addition we have noticed vast differences in the response of endothelial cells to *R. rickettsii* versus *R. conorii*. Specifically we see a delayed loss of endothelial integrity during *R. conorii* infection when compared to *R. rickettsii*. In order to further elucidate differences between *R. conorii* and *R. rickettsii* infections in endothelial cells as to NO response, we conducted an experiment infecting SV-HCEC cells with both pathogens and mock-infected cells. In addition, monolayers of infected and non-infected cells were treated with TNF- α , IFN- γ and IL-1 β , each at a concentration of 10 ng/ml. Nitrite levels in the supernatant were measured at 24 and 48 hours post-infection (Figure 1). No differences in NO production (detected indirectly by measuring nitrite levels in supernatant) was observed among all groups at 4 hours. At 24 hours, monolayers infected with *R. rickettsii* revealed a significant increase in NO production which was further enhanced by addition of cytokines. Monolayers infected with *R. conorii* did not reveal any increase in NO production whereas monolayers infected with *R. conorii* and stimulated with cytokines revealed an increase in NO production. However, such increase was not as prominent as with *R. rickettsii*-infected monolayers. Uninfected cells stimulated with cytokines also revealed an increase in NO production but lower than *R. rickettsii*- and *R. conorii*-infected monolayers. At 48 hours, the response was essentially the same as 24 hours but levels of NO were significantly higher in all groups except for *R. conorii*-infected

cells. These results have far reaching implications in Rickettsia-related research for two reasons: Firstly, NO production in human endothelial cell lines infected with rickettsial agents has not been documented in the past. All experiments have used either HUVEC's or other cell lines not of microvascular origin. NO production from human cells infected with rickettsial agents was documented by our laboratory in a hepatocyte-derived cell line after stimulation with several cytokines in combination. Secondly, the differences in NO production in monolayers infected with *R. conorii* and *R. rickettsii* with or without cytokine stimulation are dramatic and could help explain the differences in permeability changes observed in endothelial monolayers infected with *R. conorii* and *R. rickettsii*, respectively (data presented later in this section).

Calcium signaling

During the first year of the project initial experiments for intracellular calcium signaling were performed using RBE4 cells and Fluo-3, a fluorescent molecule that increases its emission in the presence of calcium. Increased calcium levels were detected as early as 60 minutes after infection with *R. rickettsii*. Elevated levels persisted up to 24 hours after infection (11). Demonstration of calmodulin activation was also demonstrated using FRET (11). The role of calcium in endothelial permeability was studied in subsequent years by using verapamil (a calcium channel blocker), BAPTA (an extracellular calcium chelator) and dantrolene (an inhibitor of intracellular calcium release from the endoplasmic reticulum). Each of the calcium blockers was added to SV-HCEC monolayers 1 hour before infection after which the medium was replaced with either regular medium or medium containing the calcium

blocker. Pre-treatment of the monolayers with verapamil followed by infection and discontinuation of the calcium blocker did not reveal major differences when compared to rickettsia-infected monolayers without calcium blockers. Infection of the monolayers followed by continued use of verapamil reveal a 5-10% increase in permeability across the monolayer, suggesting that blocking entrance of calcium from the extracellular fluid might interfere with normal homeostasis of junctional proteins (14). This observation was further confirmed by continuous addition of dantrolene to the medium, which induced a further 30-35% increase in permeability at 30 hours post-infection when compared to rickettsia-infected monolayers without dantrolene. Pre-treatment of the monolayers with dantrolene for 1 hour followed by replacement of medium without dantrolene after infection of the monolayer also increased permeability by up to 5% when compared to rickettsia-infected monolayers.

Cytokines

Effects of IL-1 β , IFN- γ and TNF- α on non-infected SV-HCEC monolayers

Cell monolayers were treated with 0.1, 1, 10, 100 and 1,000 ng/ml of TNF- α , IFN- γ , and IL-1 β . The three cytokines were used singly and in combinations. Evaluation of permeability of rickettsiae-infected monolayers in the presence of cytokines was done by infecting cell monolayers with 10 MOI and the addition of the above mentioned cytokines (16).

Increases in permeability in non-infected SV-HCEC monolayers were dose-dependent when TNF- α was added to the supernatants at concentrations ranging from 0.1 ng/ml to 1,000 ng/ml (Figure 2). At 0.1 ng/ml a mild increase in permeability (5%) was observed during the first 12 hours of infection, after which the monolayer

progressively recovered its baseline resistance at 30 hours. At concentrations of 1 ng/ml of TNF- α , a steady increase in permeability was observed which peaked at 50 hours (25% increase) and remained stable thereafter. A minor recovery (less than 5%) was observed towards the end of the experiment (72 hours). At concentrations of 10 ng/ml, a steady increase in permeability was also observed although more pronounced than the increase observed at 1 ng/ml. At 8 hours, an increase of 18% was present which reached 40% by the end of the experiment at 72 hours. At concentrations of 100 and 1000 ng/ml, a very marked increase in permeability was observed at 8 hours reaching 30 and 45%, respectively. After 8 hours, a steady increase occurred reaching 60 and 70% respectively at 72 hours.

The effects of IL-1 β on SV-HCEC monolayers were also dose-dependent, although the effects on the monolayers were less pronounced when compared to TNF- α (Figure 3). Likewise, the differences in permeability amongst all concentrations were less “linear” when compared to TNF- α . At all concentrations of IL-1 β , a rapid decline was observed at 10 hours, which ranged from 20-30%. A steady recovery of the monolayer’s resistance (decreased permeability) was observed at doses of 0.1 ng/ml, reaching control values at 72 hours. However, monolayers infected with higher doses showed a steady increase in permeability which reached values between 30-50% at concentrations of 1 ng/ml to 1,000 ng/ml, respectively.

When IFN- γ was added to SV-HCEC monolayers, minor increases in resistance (decreased permeability) were observed and ranged from 2-10% throughout the experiment. No dose dependent effect was observed in any of the monolayers (Figure 4).

Effects of TNF- α , IFN- γ , and IL-1 β on rickettsia-infected SV-HCEC monolayers

Monolayers infected with *R. rickettsii* and treated with TNF- α showed a further increase in permeability when compared to non-treated rickettsia-infected monolayers and monolayers treated with TNF- α alone in the absence of infection (Figure 5). The increase in permeability was dose-dependent at all time points. Increases in permeability were almost identical for monolayers treated with 0.1 and 1 ng/ml. In non-infected monolayers, 0.1 ng/ml of TNF- α induced a 10-12% increase in permeability at 6 hours which eventually disappeared at 30-32 hours post-treatment. However, the permeability in infected monolayers treated with 1 ng/ml of TNF- α was increased by 12% at 6 hours (as opposed to 4% in infected and non-treated monolayers). At 12 and 24 hours the differences between treated and non-treated infected monolayers were 12 and 8%, respectively. By 48 hours post-treatment, no differences were noted between TNF- α treated and non-treated monolayers. At TNF- α concentrations of 10 and 100 ng/ml, the effects were more pronounced than at lower concentrations in infected and non-infected monolayers.

Monolayers infected with *R. rickettsii* and treated with IL-1 β showed similar results to the ones obtained with TNF- α , although the effects were less dose-dependent when compared to TNF- α (Figure 6). When IFN- γ was added to the monolayers the effect was that of “stabilization” of the monolayer (Figure 7). At all doses, the infected monolayers showed decreased permeability (increased resistance). Cytokines used in different combinations (doses of 0.1 ng/ml) in infected monolayers induced an

increase in permeability that could not be neutralized by the presence of IFN- γ . No major differences were observed among the different combinations (Figure 8).

Effects of rickettsiae on SV-HCEC monolayers

SV-HCEC monolayers were infected with 1, 10, 20, and 50MOI of *R. rickettsii* and the electrode arrays were then connected to the ECIS detection system. Confluent SV-HCEC cells infected with *R. rickettsii* exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in resistance (Figure 9). Resistance recorded using SV-HCECs declined steadily over time after rickettsiae were internalized. At 24 hours, increases in permeability ranged from 12% at 1 MOI to 25% at 50 MOI. At 48 hours post-infection, monolayers infected with 1 MOI showed a 25% increase in permeability. During the first 24 to 48 hours increases in permeability were more pronounced in monolayers infected with 20 and 50 MOI as opposed to 1, 5 and 10 MOI. Differences between these two groups were less evident at 72, 96 and 120 hours of infection, most likely reflecting similar cell death rates in all monolayers at late time points. By the end of the experiment at 120 hours, all monolayers showed a 50-55% increase in permeability when compared to the controls (16).

Cell death rates in rickettsiae-infected SV-HCEC monolayers

In order to ascertain the role of cell death in increased permeability across the endothelial monolayers death cell curves were performed. SV-HCEC monolayers were infected with 15 MOI of *R. rickettsii*. At 24, 48 and 72 hours, the supernatants were aspirated and the monolayers were stained using the Live-Dead Viability Stain (Molecular Probes, Eugene, OR). Images from fields were obtained using an FV-

1000 Confocal Microscope and a 10x objective. Propidium iodide (PI) uptake by cells was used to calculate the percent of cells that were undergoing necrotic cell death. Quantification of cell death was performed by determining the percent of total cells staining positive for PI. Cell death rates did not reveal any differences between infected and non-infected monolayers up to 48 hours post-infection suggesting that increases in permeability seen during the first two days of infection are not due to cell death in the monolayers (16).

Experiments performed with Luminex technology have also revealed high concentrations of IL-6 and MCP-1 in *R. rickettsii*-infected monolayers in addition to the three cytokines that we have studied in previous years. Such increase was observed as early as 24 hours post-infection. Because of the effect on permeability that we demonstrated as caused by supernatants obtained from rickettsia-infected monolayers, we decided to use monoclonal antibodies against IL-6 and MCP-1 in order to assess their effect on endothelial permeability. However, no differences were observed between monolayers treated with supernatants with or without monoclonal antibodies against IL-6 and MCP-1, suggesting that other soluble factor might be involved in increasing endothelial permeability (14). Experiments performed with *R. conorii* reveal that the effects on endothelial permeability are attenuated. In fact, permeability barely changes during the first 48-72 hours after infection. These major differences would certainly explain the dramatically different mortality rates of *R. rickettsii* infections in humans and *R. conorii* infections. Without antibiotic treatment, the case-fatality ratio for *R. rickettsii* infections is around 30-40% as opposed to ~5% in *R. conorii* infections.

Human Endothelial Array (Oligo GEMArray® System):

In view of the dramatic differences observed between *R. conorii* and *R. rickettsii* infections both *in vitro* and *in vivo*, we decided to elucidate the response on microvascular endothelial cells after infection with both agents. The endothelial array system used in these experiments allows us to detect changes in expression of 113 genes in endothelial cells.

SV-HCEC cells were grown to confluency and infected with 10 MOI of *R. conorii* and *R. rickettsii*. One sample was used as control. Total RNA was isolated after 24 hours post-infection, directly from the culture plates containing roughly 5×10^6 SV-HCECs and desalted by ethanol precipitation (RNAqueous-4 PCR, Ambion). RNA quality was verified by UV spectrophotometry and ribosomal RNA band integrity. The TrueLabeling-AMP™ 2.0 kit was utilized to create cRNA (SuperArray). Briefly, 3ug of total RNA was added to the cDNA synthesis reaction and incubated at 42°C for 50 minutes followed by 75°C for 5 minutes then cooled to 37°C according to the manufacturers recommendations. The cRNA was then created by incubating overnight at 37°C with 10mM biotin-11-UTP (Perkin Elmer). The cRNA was then purified from the remaining reaction components using the cRNA Cleanup Kit (SuperArray). The quality and quantity of cRNA was then determined utilizing UV spectrophotometry. A total of 4ug of cRNA was hybridized to Endothelial Biology Oligo GEMArrays in a hybridization oven at 60°C overnight at 5-10rpm (SuperArray). Following several wash steps the arrays were incubated with alkaline phosphatase-conjugated streptavidin for exactly 10 minutes. The arrays were washed again and reacted with CDP-Star for 3 minutes and exposed to Hyperfilm™ ECL (Amersham

Biosciences). The developed film was captured electronically and downloaded into the GEM Express Analysis Suite. Significant differences were observed in several mRNA's including apoptosis-related genes (BCL2-like1 and BCL2-associated X protein or BAX), Ras homolog gene family member B, Tissue factor pathway inhibitor, thrombospondin 1, and TNF receptor superfamily member 10c (see table). Previous experiments performed with *R. rickettsii*-infected monolayers have shown decreased apoptotic rates mediated via NFκ-B activation. Changes in BCL-2 related genes would open a new avenue of investigation to study apoptosis-related events in infected monolayers. Thrombospondin 1 is an 420 kDa trimeric glycoprotein secreted by several cell types including endothelial cells and influences their function. One of the effects is the tyrosine phosphorylation of adherens junction proteins that regulate endothelial paracellular permeability. Marked downregulation of this mRNA certainly merits further investigation. Downregulation of the tissue factor pathway inhibitor would also help explain some of the coagulation cascade products and fibrinolytic pathway changes observed *in vitro* and *in vivo* models of rickettsial infections. Finally, marked downregulation of the Ras homolog B (Rho B) is another exciting finding in our research. This endosomal GTP-ase has received tremendous attention in cancer research because of its possible role in limiting cell proliferation, survival, invasion and metastases. Levels are commonly attenuated during malignant progression. As for the role of Rho B in rickettsial research, virtually nothing is known about its possible role in such infections. One of its functions in eukaryotic cells is in regulation of cytokine trafficking (modulation of trafficking of cell surface receptors) and cell survival.

Immunofluorescence of junctional proteins in endothelial monolayers.

We concentrated our efforts on adherens junctions and tight junctions. Experiments aimed at studying tight junctions were complicated due to the fact that microvascular endothelial cell lines do not express either occludin or claudin, the two main components of tight junctions. We therefore decided to use primary murine microvascular endothelial cells. A protocol for such purpose was developed. Expression of occludin and ZO-1 were demonstrated by confocal microscopy (14). Experiments with the primary mouse microvascular endothelial cells (MBEC) infected with *R. rickettsii* showed a frayed appearance of occludin staining after 24 hours of infection (Figure 10).

Experiments using SV-HCEC demonstrated loss of p120/ β -catenin staining at intercellular junctions as early as 12 hours after infection with formation of granular deposits of p120 in the cytoplasm and formation of intercellular gaps (Figure 11). These changes correlate well with the changes observed in vascular permeability using ECIS. Infection with *R. conorii* did not have the same effect on p120/ β -catenin at up to 72 hours of infection. Expression of p120 did not reveal any changes by western immunoblotting, implying post-translational modification.

III. KEY RESEARCH ACOMPLISHMENTS

1. Construction of more than two dozen libraries using plasmid ptatCCDlib- β -gal and ptatCCD-EGFP encoding combinatorial 6-mer, 12-mer and 18-mer adapteins with $\sim 2 \times 10^7$ - $3-5 \times 10^8$.

2. Large scale production of combinatorial libraries in bacterial cells.
3. Challenge experiments with rickettsial pathogens were not successful in expanding resistant clones or cell colonies. We continue to experiment under different conditions and with other cell lines. Libraries are continuously available.
4. Successful infection of murine and human monocytic cell lines with adaptein-containing retroviruses for rickettsial challenges with cell suspensions
5. Acquisition of several rat, mouse and human microvascular endothelial cells for experiments involving vascular permeability (SV-HCEC, TIME, RBE4).
6. Development of a protocol to isolate primary brain microvascular endothelial cells of murine origin.
7. Development of a reliable, continuous, and reproducible system to study microvascular endothelial permeability on a continuous basis using Electronic Cell Substrate Impedance Sensing in the Biosafety Level 3 environment. This technique can be applied to any pathogen capable of increasing microvascular or epithelial permeability. In fact, several collaboration have resulted with researchers on campus working with viral hemorrhagic fever viruses.
8. Demonstration of the role of cytokines (IL-1 β , IFN- γ , and TNF- α) in endothelial permeability during infection with *R. rickettsii*.
9. Demonstration of the different effects of two pathogenic rickettsiae (*R. conorii* and *R. rickettsii*) in endothelial permeability.
10. Demonstration of the role of ROS and NO as rickettsicidal mechanisms and as factors affecting endothelial permeability.

11. Demonstration of the role of p120/ β -catenin in adherens junctions changes in SV-HCEC monolayers.
12. Demonstration of the role of occluding in tight junction changes in primary murine brain endothelial cells.
13. Demonstration of differential regulation of five genes *R. conorii*- and *R. rickettsii*-infected microvascular endothelial cells using mRNA microarrays.

IV. REPORTABLE OUTCOMES

Abstracts and Presentations

1. Olano JP. Pathology of selected and potential bioterrorist agents. Houston Society of Clinical Pathology. 2002.
2. Olano JP, Wen G. Evidence of calmodulin activation in an in vitro model of rickettsial infection. American Society of Investigative Pathology. FASEB J. 2002;16:A202.
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Publications in peer-reviewed journals

1. Woods ME, Koo P, Wen G, Olano JP. Nitric oxide (NO) as a mediator of increased microvascular permeability during rickettsial infections. Annals of the New York Academy of Sciences. 2005;1063:239-245.
2. Olano JP. Rickettsial infections. Annals of the New York Academy of Sciences. 2005;1063:187-196.
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Technical Reports

1. Olano JP, Walker DH. Molecular Pathogenesis of Rickettsioses and Development of Novel Anti-Rickettsial Treatment by Combinatorial Peptide-Based Libraries. Technical Reports submitted to the United States AMRMC. 2003, 2004, 2005, 2006.

Manuscripts submitted/in preparation

1. Woods ME, Olano JP. Host defenses to *Rickettsia rickettsii* infection contribute to increased microvascular permeability in human cerebral endothelial cells. American Journal of Pathology (draft attached).
2. Woods ME, Olano JP. Differences in endothelial permeability in *Rickettsia conorii*- and *Rickettsia rickettsii*-infected human brain microvascular endothelial cells: Pathogenetic implications for human disease. American Journal of Pathology (in preparation).

Personnel trained

1. Michael E. Woods, B.Sc. Mr. Woods performed all experiments related to his Ph.D. dissertation. He will defend his thesis in the fall of 2007.

Grant applications

We are planning to submit two grant applications: the first one will concentrate on proteomics of microvascular endothelial cells and its use in prognosis of patients acutely infected with rickettsiae. Endothelial permeability markers will be analyzed. The second project will be in collaboration with Sandia National Laboratories and will concentrate on the creation of highly advanced and automated in vitro models of the blood brain barrier and alveolo-capillary barrier in the lungs. Both projects will use large amounts a data generated by this contract.

V. CONCLUSIONS

The role of rickettsial organisms alone and in combination with cytokines (TNF- α , IFN- γ and IL-1 β) in endothelial permeability is now well defined. We have demonstrated a synergistic effect of rickettsiae and pro-inflammatory cytokines in

negatively regulating the integrity of endothelial monolayers. Specifically, we have shown that very low doses of TNF- α , IL-1 β or IFN- γ (0.1 or 1ng/ml) either singly or in combination, coupled with *R. rickettsii* infection leads to a significant increase in endothelial permeability. The response to cytokine stimulation is dose dependent in the absence of rickettsial infection as is the effect of rickettsial infection alone. This cytokine stimulation is not accompanied by an increase in nitric oxide production in the SV-HCEC cell line. However, minor increases in NO production have been documented in SV-HCEC infected with *R. rickettsii*. Such increases probably are not high enough to affect endothelial permeability. The role of NO in controlling rickettsial infections in mice has been very well documented. However, its role in humans remains controversial. For human cells to increase NO production, several cytokines have to be used in combination as documented by in vitro experiments.

Experiments demonstrate that TNF- α has the greatest impact on rickettsiae-infected cells resulting in a 15% increase in permeability when compared to infected cells even at doses as low as 0.1ng/ml. The synergistic effects of IL-1 β do not seem to be as pronounced in infected cells when compared to those of TNF- α . Likewise we have demonstrated the loss of p120/catenin staining at endothelial junctions as early as 12 hours post-rickettsial infection with *R. rickettsii*. The regular staining pattern of cell borders becomes fragmented and disjointed and antedates the formation of intercellular gaps. These changes correlate well with the increased permeability observed by ECIS. Conversely, in monolayers infected with *R. conorii* we did not observe such changes in p120/ β -catenin (Figure 10). Expression of p120 as measured by western blot suggesting a post-translational modification is occurring, perhaps in the form of tyrosine

phosphorylation.

Infection of endothelial monolayers with *R. conorii* has no effect on endothelial permeability for the first 2-3 days of infection, after which time the cells succumb to the high level of infection. Current experiments are aimed at delineating the origin of increased virulence of *R. rickettsii* through gene expression arrays and proteomic analysis of host responses to infection. The Oligo GEArray[®] System Human Endothelial Array allowed us to detect changes in expression of five genes associated with endothelial cell biology and has shown us potentially important differential regulation in *R. rickettsii* versus *R. conorii*-infected monolayers. This important information is currently being used to generate data by using quantitative RT-PCR targeting those specific genes at different time points of infection (6, 12, 24, 48 and 72 hours). In addition, the ProteomeLab[™] PF 2D system will allow us to systematically investigate the host proteome response to rickettsial infection in a highly sensitive, reliable and automated manner. We expect this to provide us with high amounts of information about the host response to rickettsial infection leading to many important avenues of research. The results of these experiments will be reported as an addendum.

VI. SCIENTIFIC PERSONNEL

1. David H. Walker, Principal Investigator(10% effort)
2. Juan P. Olano, M.D. Co-Principal Investigator (25% effort)
3. Stanley Watowich, Ph.D., Collaborator (first two years of the award, 10% effort)
4. Robert A. Davey, Ph.D. Collaborator (first two years of the award, 10% effort).
5. Paul Koo, Ph.D. Post-doctoral fellow (2002-2005)
6. Olga Kolokoltsova. Research Technician (2002-2004)

7. Drew Deniger. Research Technician (2002-2004
8. Gary Wen, M.Sc. Research Associate (2004-2007)
9. Michael E. Woods, B.Sc. Graduate student (100% effort, 2004-2007)
10. Leoncio Vergara, M.D. (10% effort, June 2004-Feb 2006)

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19. Woods ME, Olano JP. Peroxynitrite-mediated damage during rickettsial infection of human microvascular endothelial cells. American Society of Investigative Pathology. San Francisco. 2006.

Figure 1

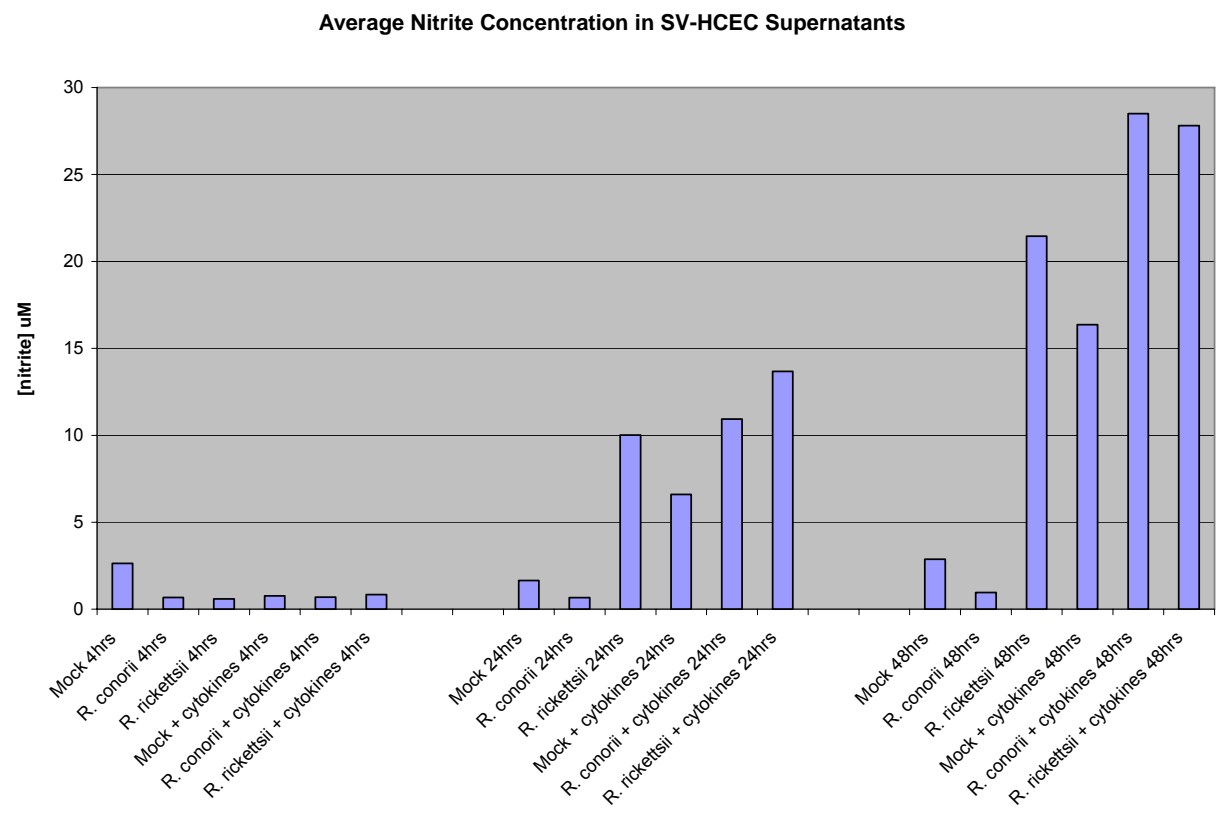


Figure 2

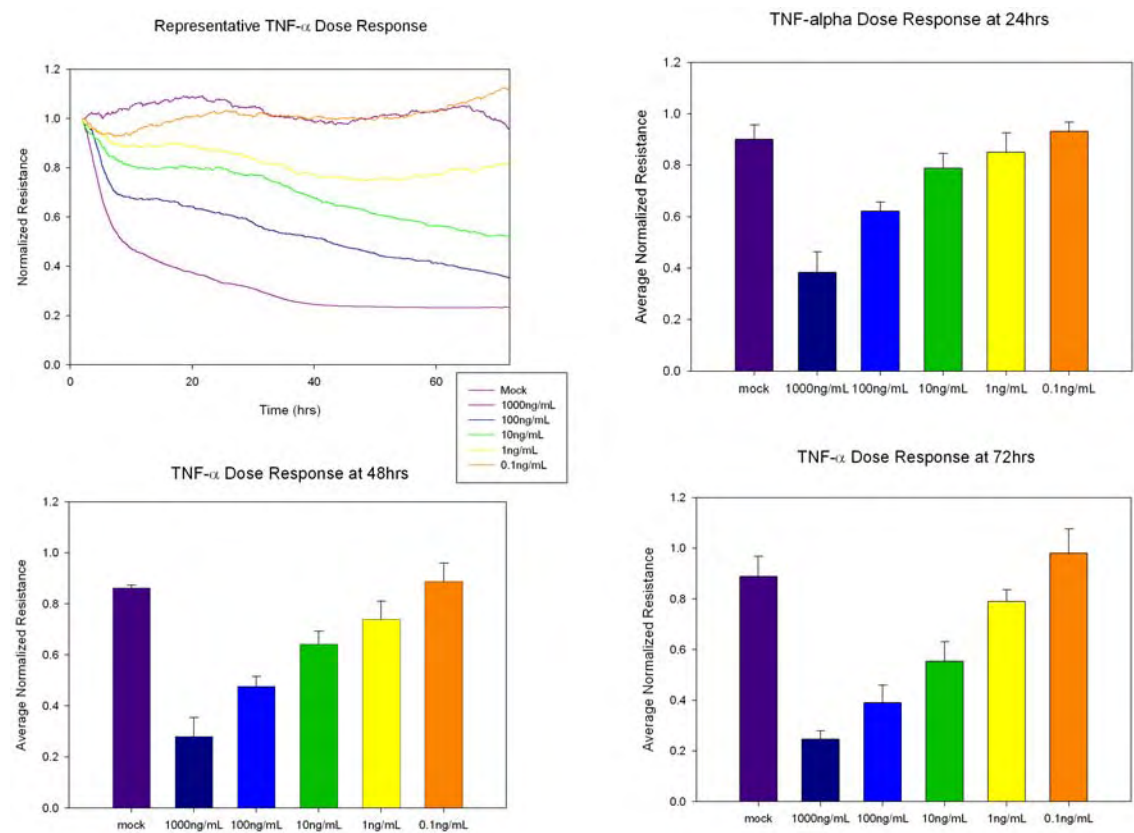


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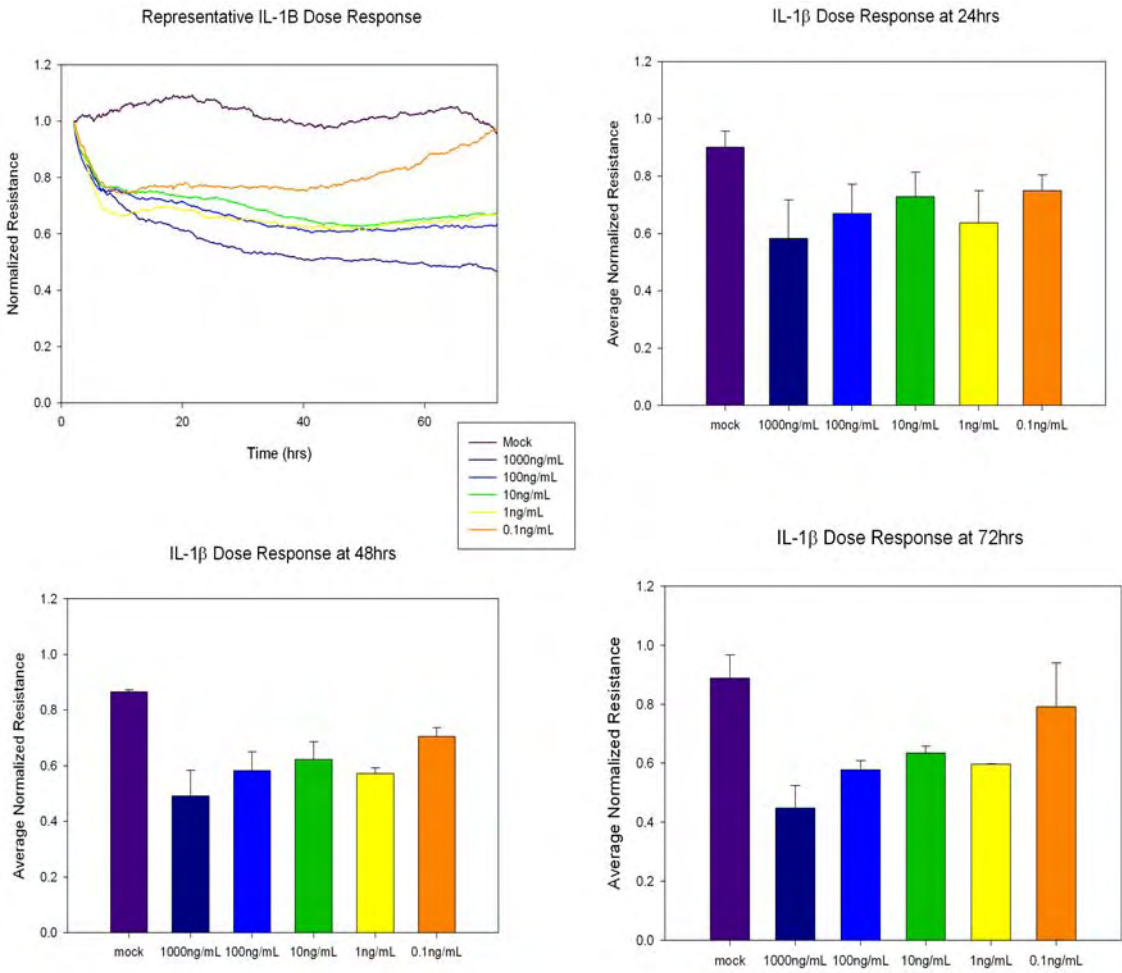


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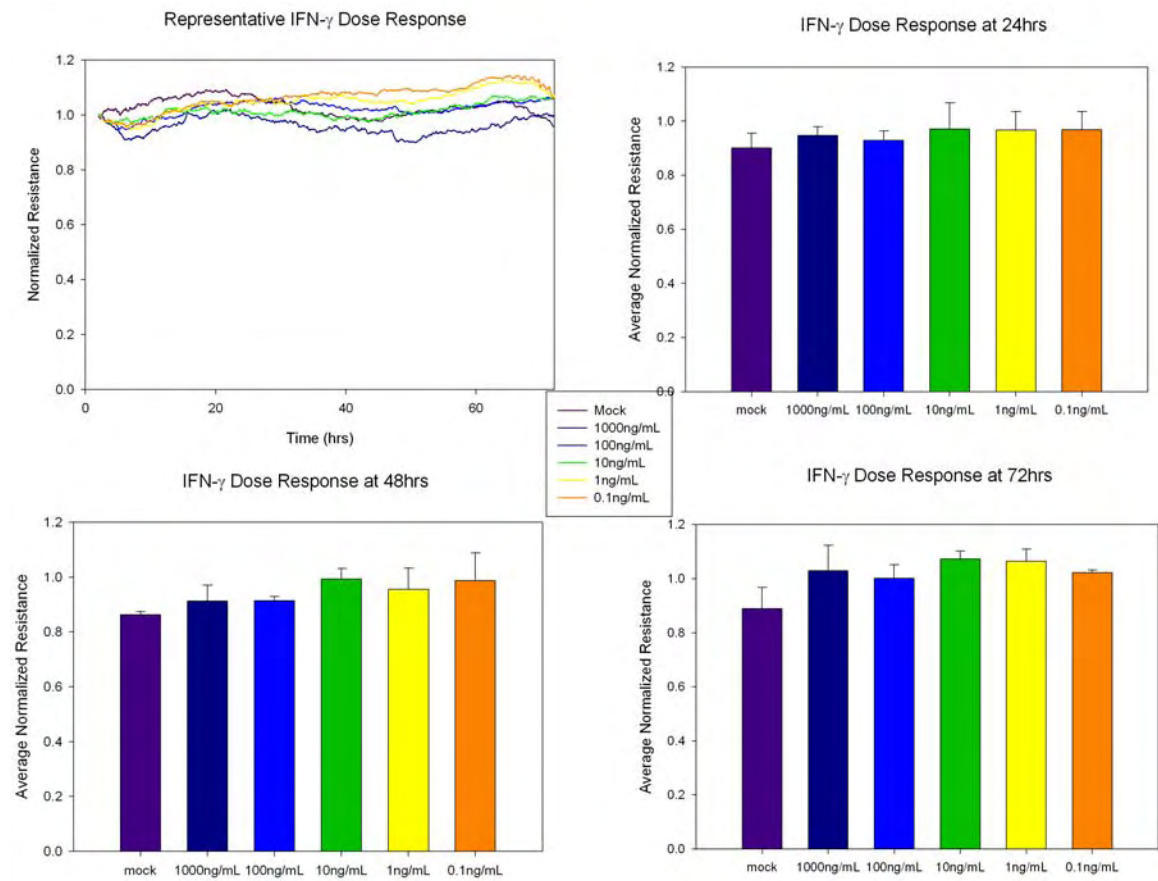


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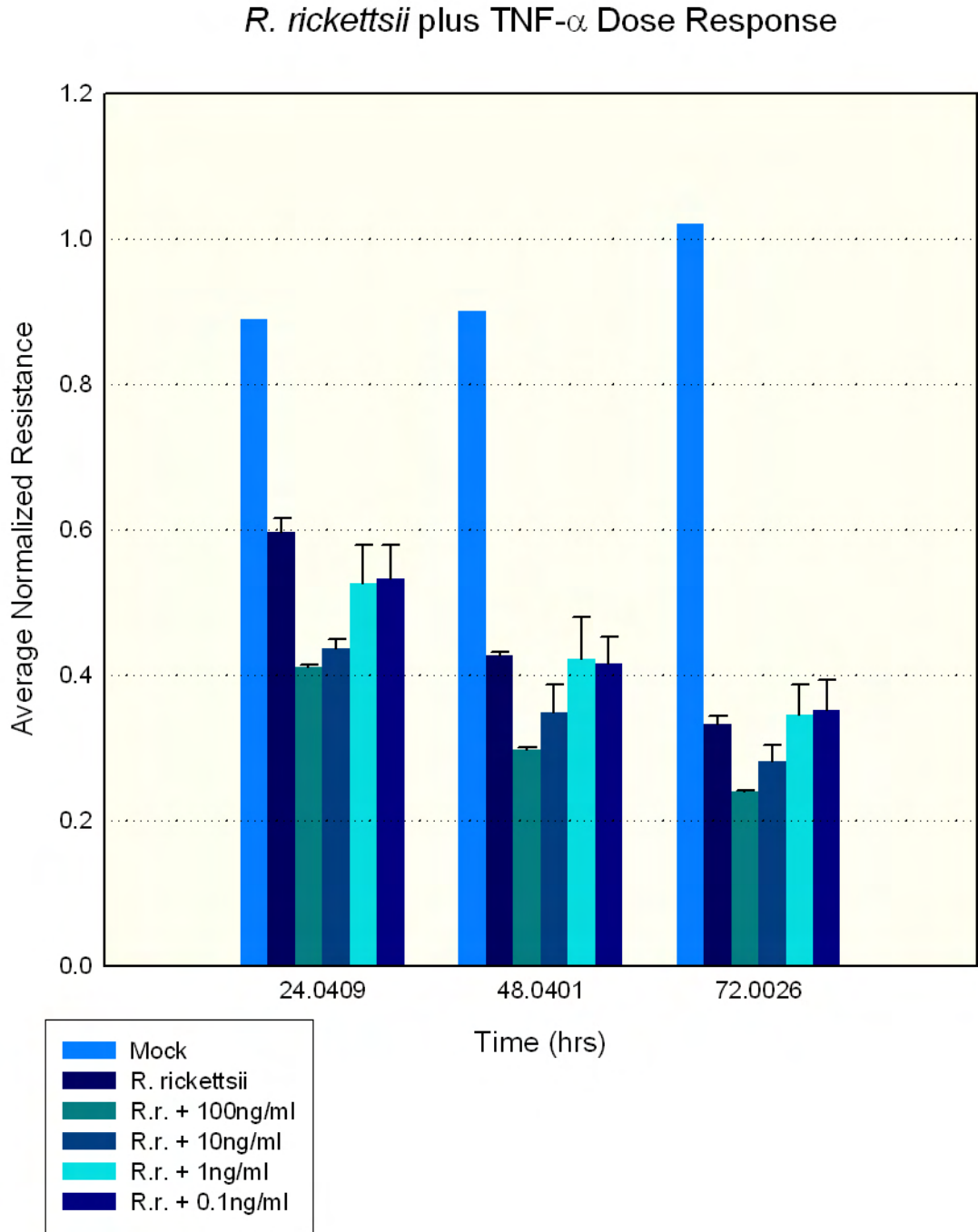


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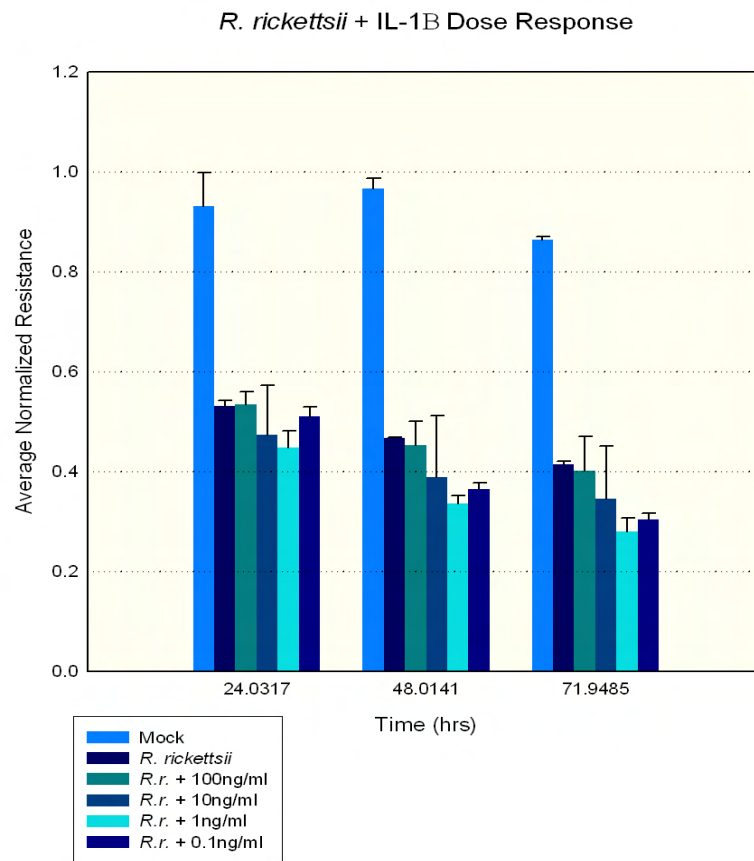


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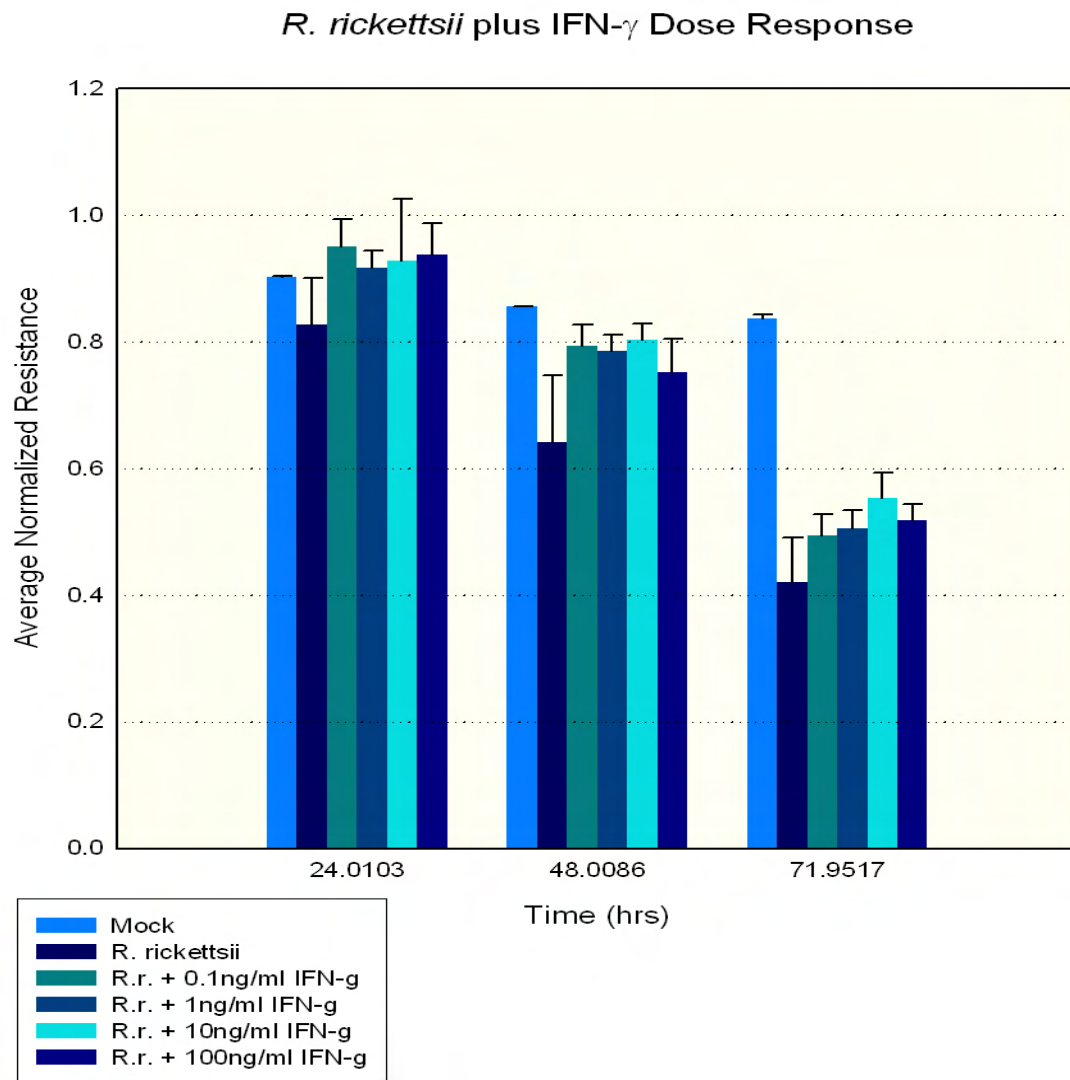


Figure 8

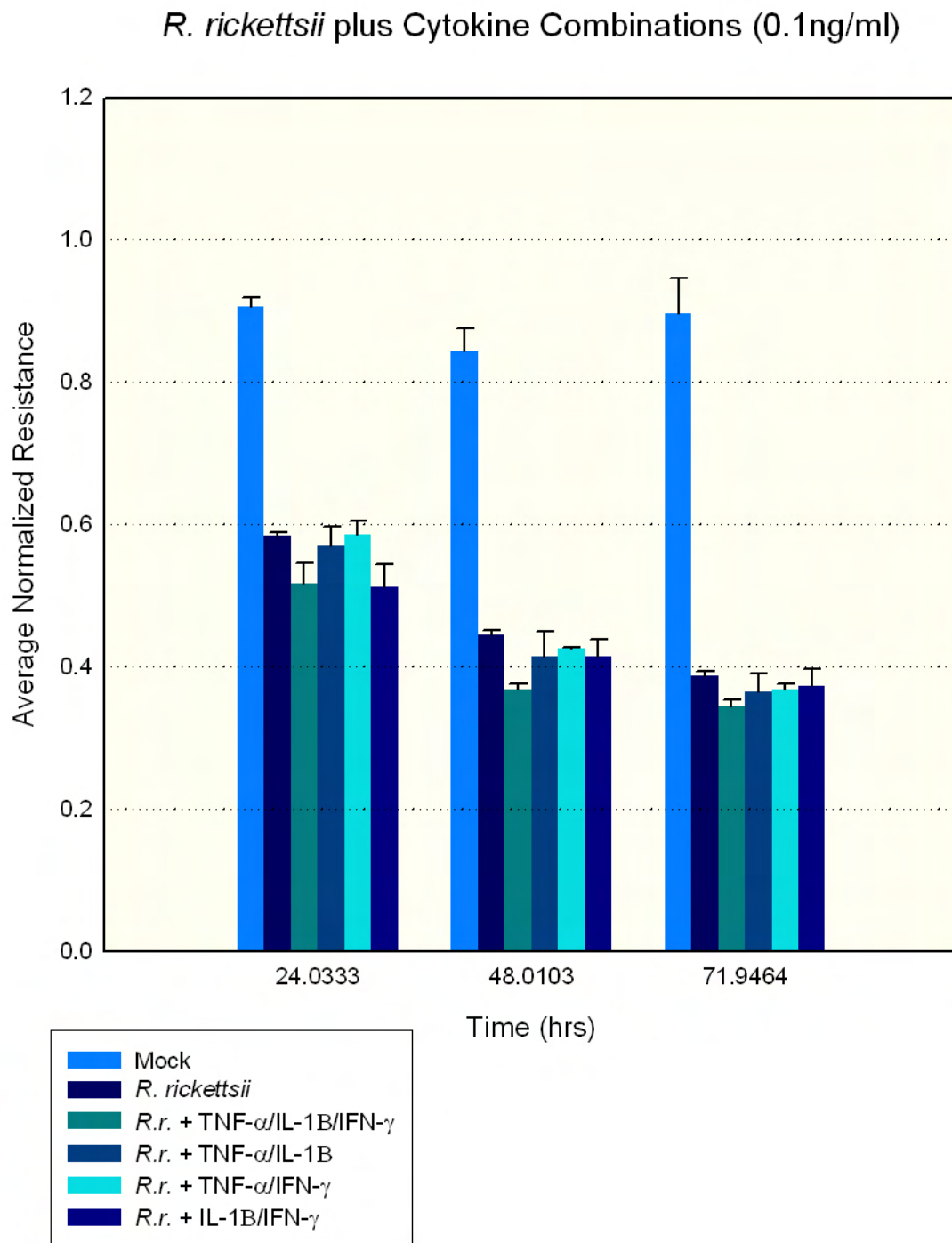


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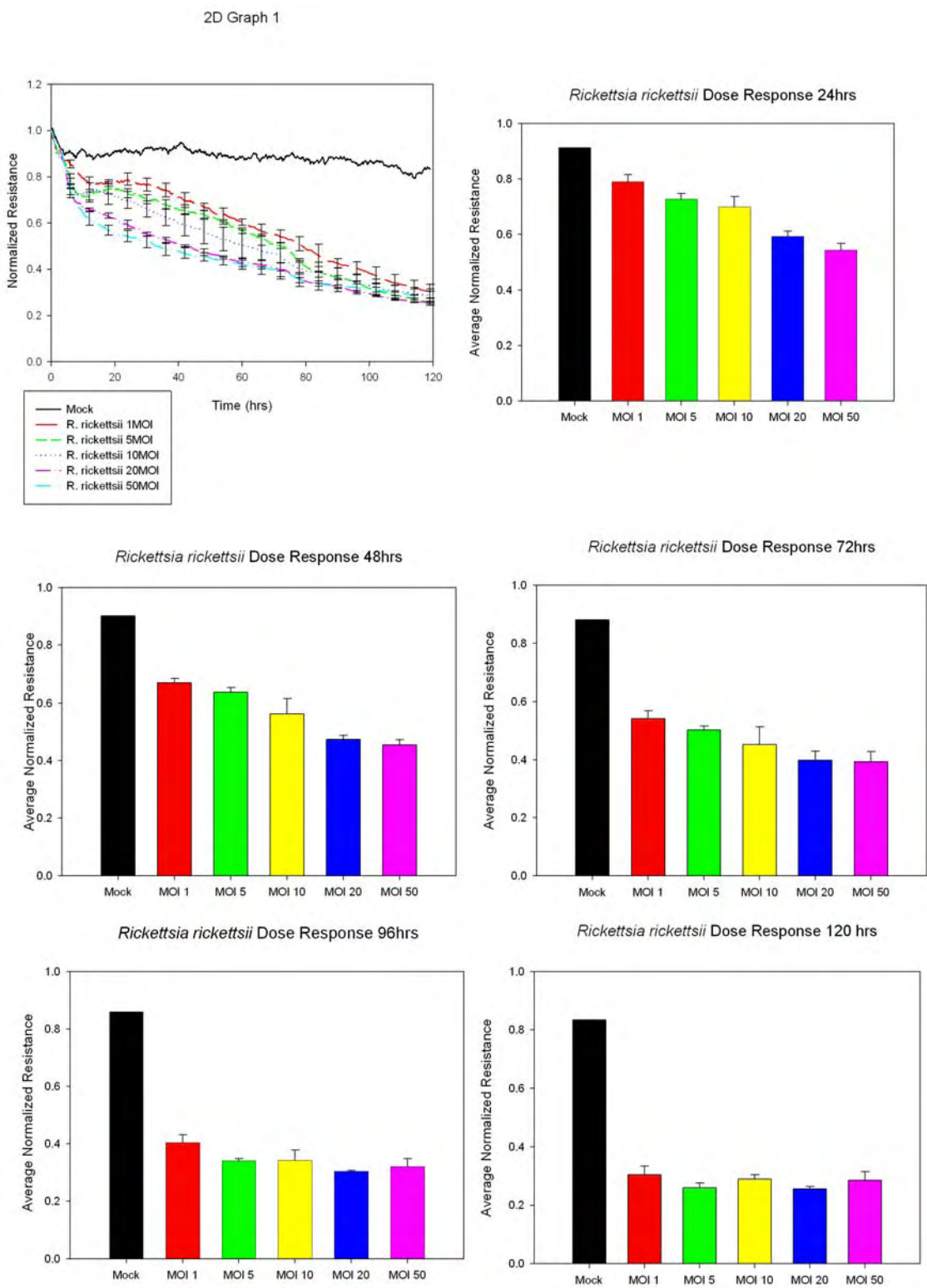


Figure 10

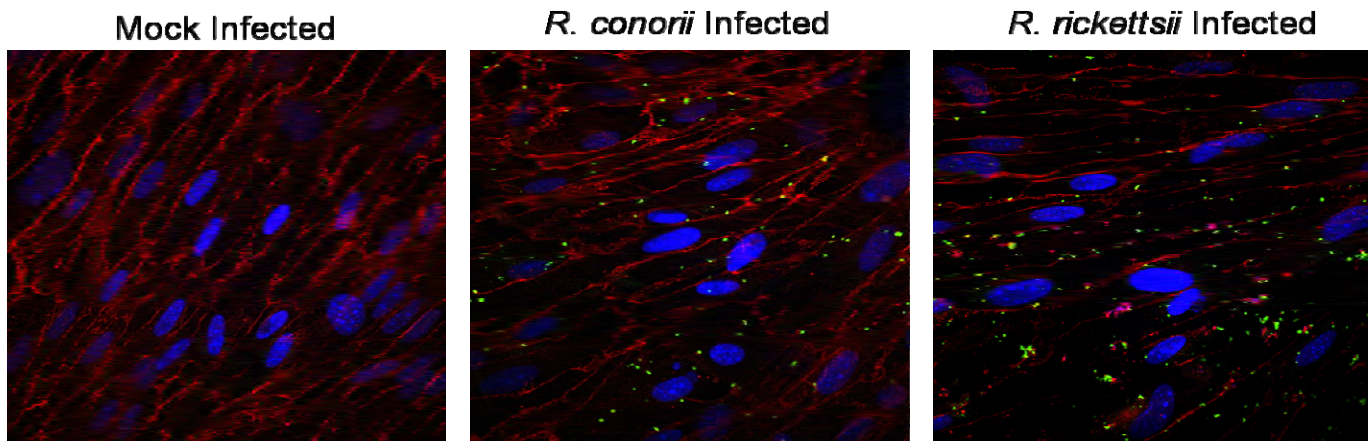
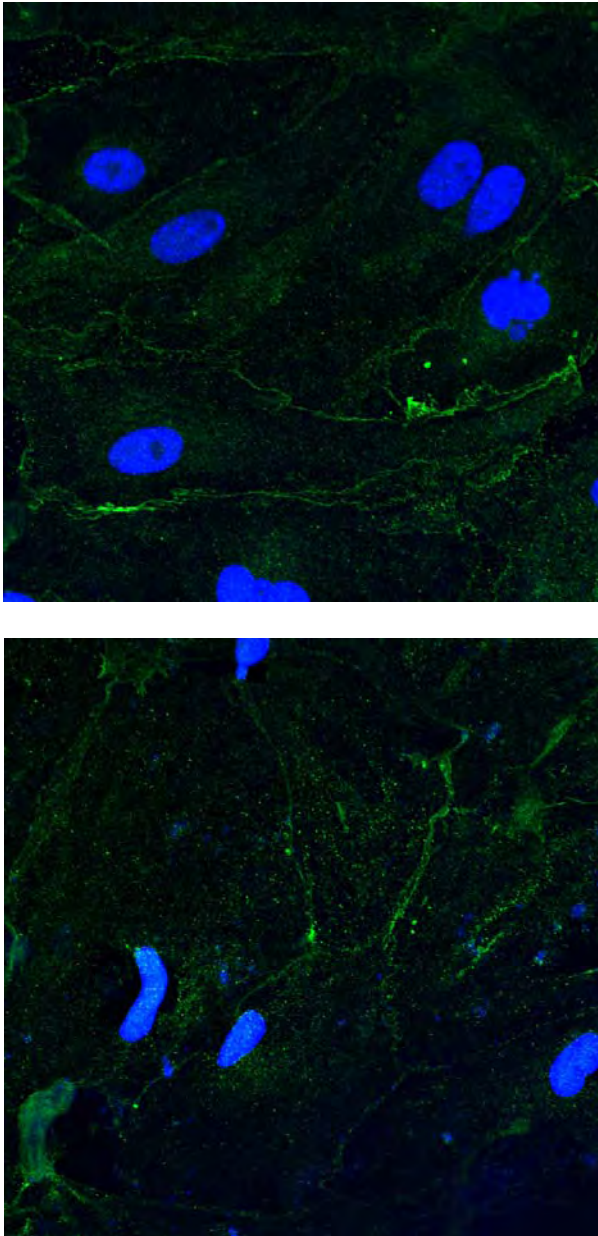


Figure. MBECs (p0) were infected with rickettsiae and fixed in methanol at 24 hours after infection. The cells were then stained with mouse anti-p120 and rabbit anti-SFG antibodies. p120 is in red, rickettsiae is in green

Figure 11



Primary murine brain microvascular endothelial cells stain with anti-occludin antibodies. Top: Mock infected at 24 hours. Bottom: Infected with *R. rickettsii* for 24 hours. Note “frayed” appearance of occluding membrane staining in infected cells. DAPI was used as nuclear counterstain.

TABLE

Project SV-HCEC rickettsiae
 Catalog # Oligo GEM Array Human Endothelial Cell Biology Microarray,
 Density Average
 Clover Clover On
 Total Arrays 6
 Total Groups 3
 Background Empty spots: positions
 AP threshold 1.20
 Normalization Selected genes: positions 1, 127, 128

Array Name	Mock 1min exposure	R. conorii 1min exposure	R. rickettsii 1min exposure	Mock 30sec exposure	R. conorii 30sec exposure	R. rickettsii 30sec exposure
Assigned Group	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3

* As default, Group 1 is used as control

Numbers reported BLUE indicate that these spots are considered as "Absent" (see Page "Absent_Present Calls" for details).

Numbers reported RED indicate that these spots are considered as "Bleeding" (see Page "Bleeding" for details).

Position	UniGene	RefSeq Number	Symbol	Description	Group 1			Group 2			Group 3			Group 2/ Group 1	Group 3/ Group 1
					Mock 1min	Mock	average	R. conorii 1min	R. conorii	average	R. rickettsii 1min	R. rickettsii	average		
1	Hs.311640	NM_002954	RPS27A	Ribosomal protein S27a	0.80	0.58	0.69	0.82	0.62	0.72	0.60	0.38	0.49	1.05	0.72
2	Hs.298469	NM_152831	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	0.07	0.00	0.04	0.28	0.15	0.21	0.05	0.03	0.04	5.95	1.22
3	Hs.178098	NM_021804	ACE2	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	-0.16	-0.10	-0.13	-0.05	-0.01	-0.03	-0.07	-0.05	-0.06	0.24	0.43
4	Hs.404914	NM_003183	ADAM17	ADAM metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)	-0.17	-0.09	-0.13	-0.01	-0.02	-0.01	-0.07	-0.03	-0.05	0.10	0.36
5	Hs.19383	NM_000029	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	-0.22	-0.14	-0.18	-0.08	-0.04	-0.06	-0.08	-0.03	-0.05	0.35	0.30
6	Hs.477887	NM_031850	AGTR1	Angiotensin II receptor, type 1	-0.22	-0.13	-0.18	-0.06	-0.03	-0.05	-0.09	-0.05	-0.07	0.27	0.39
7	Hs.405348	NM_000686	AGTR2	Angiotensin II receptor, type 2	-0.20	-0.14	-0.17	-0.09	-0.05	-0.07	-0.10	-0.05	-0.08	0.41	0.44
8	Hs.89499	NM_000698	ALOX5	Arachidonate 5-lipoxygenase	-0.19	-0.11	-0.15	-0.08	-0.06	-0.07	-0.08	-0.04	-0.06	0.47	0.40
9	Hs.369675	NM_001146	ANGPT1	Angiopoietin 1	-0.14	-0.11	-0.12	0.00	-0.01	0.00	-0.03	-0.01	-0.02	0.02	0.17
10	Hs.553484	NM_001147	ANGPT2	Angiopoietin 2	-0.17	-0.13	-0.15	0.00	0.00	0.00	-0.04	-0.02	-0.03	0.00	0.18
11	Hs.209153	NM_014495	ANGPTL3	Angiopoietin-like 3	-0.14	-0.07	-0.11	0.04	0.03	0.03	-0.02	0.00	-0.01	-0.31	0.08
12	Hs.480653	NM_001154	ANXA5	Annexin A5	0.97	1.18	1.07	1.05	1.23	1.14	1.15	1.23	1.19	1.07	1.11

13	Hs.72885	NM_001700	AZU1	Azurocidin 1 (cationic antimicrobial protein 37)	-0.07	-0.06	-0.07	0.11	0.07	0.09	0.01	0.00	0.00	-1.32	-0.05
14	Hs.159428	NM_004324	BAX	BCL2-associated X protein	0.74	0.54	0.64	0.99	0.99	0.99	0.66	0.44	0.55	1.55	0.85
15	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2	-0.15	-0.09	-0.12	-0.01	-0.01	-0.01	-0.04	-0.02	-0.03	0.05	0.27
16	Hs.227817	NM_004049	BCL2A1	BCL2-related protein A1	-0.16	-0.08	-0.12	-0.08	-0.05	-0.07	-0.09	-0.04	-0.06	0.55	0.52
17	Hs.516966	NM_138578	BCL2L1	BCL2-like 1	1.01	1.05	1.03	1.02	1.03	1.03	0.65	0.41	0.53	1.00	0.52
18	Hs.113916	NM_001716	BLR1	Burkitt lymphoma receptor 1, GTP binding protein (chemokine (C-X-C motif) receptor 5)	0.82	0.60	0.71	0.84	0.64	0.74	0.69	0.45	0.57	1.04	0.80
19	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	0.03	-0.03	0.00	0.09	0.07	0.08	0.03	0.02	0.03	33.91	12.11
20	Hs.5353	NM_001230	CASP10	Caspase 10, apoptosis-related cysteine peptidase	-0.13	-0.12	-0.12	0.00	0.02	0.01	-0.03	-0.01	-0.02	-0.08	0.18
21	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase	-0.21	-0.12	-0.17	-0.05	-0.01	-0.03	-0.07	-0.03	-0.05	0.18	0.29
22	Hs.3280	NM_032992	CASP6	Caspase 6, apoptosis-related cysteine peptidase	-0.18	-0.11	-0.15	-0.04	-0.02	-0.03	-0.06	-0.03	-0.04	0.20	0.30
23	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2	-0.18	-0.12	-0.15	-0.04	-0.02	-0.03	-0.05	-0.02	-0.04	0.22	0.24
24	Hs.75498	NM_004591	CCL20	Chemokine (C-C motif) ligand 20	-0.17	-0.09	-0.13	-0.03	-0.02	-0.02	-0.06	-0.02	-0.04	0.17	0.28
25	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5	-0.06	-0.05	-0.05	0.07	0.03	0.05	-0.01	0.01	0.00	-0.93	0.03
26	Hs.76206	NM_001795	CDH5	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	-0.12	-0.10	-0.11	-0.01	0.00	-0.01	-0.04	-0.02	-0.03	0.05	0.27
27	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	-0.19	-0.14	-0.17	-0.03	-0.02	-0.03	-0.05	-0.04	-0.04	0.16	0.26
28	Hs.150793	NM_001275	CHGA	Chromogranin A (parathyroid secretory protein 1)	-0.23	-0.14	-0.19	-0.07	-0.03	-0.05	-0.03	-0.02	-0.03	0.26	0.15
29	Hs.517356	NM_030582	COL18A1	Collagen, type XVIII, alpha 1	-0.23	-0.14	-0.18	-0.09	-0.04	-0.07	-0.06	-0.03	-0.05	0.38	0.26
30	Hs.512937	NM_001872	CPB2	Carboxypeptidase B2 (plasma, carboxypeptidase U)	-0.14	-0.10	-0.12	-0.03	-0.01	-0.02	-0.01	-0.01	-0.01	0.17	0.09

31	Hs.38533	NM_003805	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	-0.18	-0.12	-0.15	-0.05	-0.03	-0.04	-0.05	-0.02	-0.04	0.26	0.25
32	Hs.1349	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	-0.18	-0.11	-0.14	-0.06	-0.03	-0.05	-0.06	-0.03	-0.04	0.31	0.30
33	Hs.2233	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)	0.05	0.00	0.02	0.29	0.15	0.22	0.06	0.02	0.04	8.97	1.78
34	Hs.531668	NM_002996	CX3CL1	Chemokine (C-X3-C motif) ligand 1	-0.16	-0.13	-0.14	-0.02	-0.02	-0.02	-0.03	-0.02	-0.03	0.14	0.18
35	Hs.546251	NM_001953	ECGF1	Endothelial cell growth factor 1 (platelet-derived)	-0.13	-0.11	-0.12	0.09	0.05	0.07	-0.03	0.00	-0.01	-0.59	0.12
36	Hs.511899	NM_001955	EDN1	Endothelin 1	-0.22	-0.14	-0.18	-0.07	-0.03	-0.05	-0.05	-0.02	-0.04	0.29	0.20
37	Hs.1407	NM_001956	EDN2	Endothelin 2	-0.23	-0.14	-0.18	-0.09	-0.04	-0.07	-0.05	-0.04	-0.04	0.37	0.24
38	Hs.1408	NM_000114	EDN3	Endothelin 3	-0.21	-0.12	-0.17	-0.08	-0.05	-0.07	-0.06	-0.04	-0.05	0.39	0.28
39	Hs.183713	NM_001957	EDNRA	Endothelin receptor type A	-0.20	-0.13	-0.17	-0.08	-0.05	-0.06	-0.04	-0.03	-0.04	0.38	0.24
40	Hs.82002	NM_000115	EDNRB	Endothelin receptor type B	-0.18	-0.08	-0.13	-0.07	-0.04	-0.05	-0.06	-0.02	-0.04	0.42	0.34
41	Hs.435765	NM_001977	ENPEP	Glutamyl aminopeptidase (aminopeptidase A)	-0.17	-0.11	-0.14	-0.02	-0.02	-0.02	-0.06	-0.03	-0.05	0.12	0.34
42	Hs.62192	NM_001993	F3	Coagulation factor III (thromboplastin, tissue factor)	-0.16	-0.13	-0.15	-0.04	-0.03	-0.04	-0.06	-0.03	-0.05	0.24	0.33
43	Hs.483635	NM_000800	FGF1	Fibroblast growth factor 1 (acidic)	-0.21	-0.15	-0.18	-0.04	-0.03	-0.04	-0.06	-0.05	-0.06	0.20	0.31
44	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	-0.21	-0.13	-0.17	-0.06	-0.04	-0.05	-0.05	-0.03	-0.04	0.29	0.25
45	Hs.507621	NM_002019	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	-0.21	-0.12	-0.16	-0.08	-0.05	-0.06	-0.05	-0.03	-0.04	0.38	0.26
46	Hs.507590	NM_004119	FLT3	Fms-related tyrosine kinase 3	-0.17	-0.10	-0.13	-0.07	-0.04	-0.06	-0.06	-0.03	-0.04	0.44	0.33
47	Hs.415048	NM_002020	FLT4	Fms-related tyrosine kinase 4	-0.04	-0.03	-0.03	0.09	0.05	0.07	0.00	0.01	0.01	-1.98	-0.16
48	Hs.203717	NM_002026	FN1	Fibronectin 1	-0.15	-0.07	-0.11	0.02	0.00	0.01	-0.03	0.00	-0.02	-0.08	0.16
49	Hs.515126	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	-0.15	-0.10	-0.13	-0.04	-0.04	-0.04	-0.06	-0.04	-0.05	0.32	0.39
50	Hs.431460	NM_000873	ICAM2	Intercellular adhesion molecule 2	-0.18	-0.13	-0.16	-0.04	-0.03	-0.04	-0.06	-0.04	-0.05	0.24	0.31

51	Hs.353214	NM_002162	ICAM3	Intercellular adhesion molecule 3	-0.14	-0.12	-0.13	-0.01	-0.01	-0.01	-0.03	-0.02	-0.03	0.06	0.21
52	Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast	0.11	0.03	0.07	0.13	0.06	0.10	0.07	0.03	0.05	1.35	0.72
53	Hs.467304	NM_000641	IL11	Interleukin 11	-0.19	-0.13	-0.16	-0.08	-0.03	-0.06	-0.06	-0.04	-0.05	0.34	0.28
54	Hs.17987	NM_175852	TXLNA	Taxilin alpha	-0.07	-0.05	-0.06	-0.03	-0.03	-0.03	-0.05	-0.03	-0.04	0.42	0.63
55	Hs.168132	NM_172175	IL15	Interleukin 15	0.05	0.01	0.03	0.03	0.01	0.02	-0.01	0.01	0.00	0.77	-0.03
56	Hs.126256	NM_000576	IL1B	Interleukin 1, beta	1.10	1.13	1.11	1.07	1.20	1.14	1.15	1.26	1.20	1.02	1.08
57	Hs.694	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)	-0.02	-0.03	-0.03	0.04	0.02	0.03	0.01	-0.01	0.00	-1.09	-0.02
58	Hs.512234	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	-0.19	-0.13	-0.16	-0.05	-0.05	-0.05	-0.06	-0.04	-0.05	0.31	0.30
59	Hs.536926	NM_000880	IL7	Interleukin 7	-0.20	-0.14	-0.17	-0.05	-0.04	-0.04	-0.07	-0.04	-0.05	0.25	0.30
60	Hs.624	NM_000584	IL8	Interleukin 8	-0.20	-0.13	-0.17	-0.06	-0.04	-0.05	-0.06	-0.04	-0.05	0.29	0.28
61	Hs.505654	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	-0.19	-0.12	-0.16	-0.09	-0.06	-0.07	-0.06	-0.04	-0.05	0.45	0.34
62	Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	-0.15	-0.09	-0.12	-0.03	-0.03	-0.03	-0.04	-0.04	-0.04	0.26	0.31
63	Hs.429052	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	-0.08	-0.04	-0.06	0.05	0.04	0.05	-0.02	0.00	-0.01	-0.75	0.12
64	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	-0.04	-0.03	-0.04	0.01	0.00	0.01	-0.02	0.00	-0.01	-0.21	0.34
65	Hs.479756	NM_002253	KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	-0.12	-0.09	-0.11	-0.06	-0.04	-0.05	-0.06	-0.04	-0.05	0.48	0.47
66	Hs.479754	NM_000222	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-0.16	-0.12	-0.14	-0.06	-0.04	-0.05	-0.06	-0.04	-0.05	0.36	0.37
67	Hs.171995	NM_001648	KLK3	Kallikrein 3, (prostate specific antigen)	-0.17	-0.12	-0.15	-0.06	-0.03	-0.05	-0.06	-0.05	-0.05	0.31	0.38
68	Hs.99900	NM_002377	MAS1	MAS1 oncogene	-0.21	-0.13	-0.17	-0.07	-0.05	-0.06	-0.08	-0.04	-0.06	0.37	0.35
69	Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	-0.20	-0.13	-0.16	-0.07	-0.05	-0.06	-0.07	-0.05	-0.06	0.35	0.35

70	Hs.2399	NM_004995	MMP14	Matrix metalloproteinase 14 (membrane-inserted)	-0.11	-0.07	-0.09	-0.01	0.01	0.00	-0.02	-0.02	-0.02	0.01	0.20
71	Hs.513617	NM_004530	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1.10	1.04	1.07	1.07	1.03	1.05	1.14	1.21	1.18	0.98	1.10
72	Hs.297413	NM_004994	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	-0.12	-0.06	-0.09	-0.03	0.01	-0.01	-0.01	0.00	0.00	0.10	0.05
73	Hs.462525	NM_000625	NOS2A	Nitric oxide synthase 2A (inducible, hepatocytes)	-0.08	-0.08	-0.08	0.00	0.00	0.00	-0.04	-0.03	-0.04	0.02	0.45
74	Hs.511603	NM_000603	NOS3	Nitric oxide synthase 3 (endothelial cell)	-0.15	-0.11	-0.13	-0.06	-0.04	-0.05	-0.06	-0.04	-0.05	0.37	0.36
75	Hs.219140	NM_002521	NPPB	Natriuretic peptide precursor B	-0.19	-0.14	-0.16	-0.08	-0.05	-0.07	-0.07	-0.05	-0.06	0.40	0.37
76	Hs.490330	NM_000906	NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	-0.21	-0.13	-0.17	-0.08	-0.05	-0.06	-0.08	-0.05	-0.07	0.38	0.39
77	Hs.482439	NM_002538	OCLN	Occludin	-0.18	-0.13	-0.15	-0.08	-0.06	-0.07	-0.06	-0.03	-0.05	0.45	0.32
78	Hs.74615	NM_006206	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	-0.15	-0.10	-0.13	-0.03	-0.02	-0.03	-0.05	-0.02	-0.04	0.21	0.28
79	Hs.509067	NM_002609	PDGFRB	Platelet-derived growth factor receptor, beta polypeptide	-0.09	-0.06	-0.08	-0.01	-0.02	-0.01	-0.04	-0.02	-0.03	0.15	0.38
80	Hs.514412	NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	-0.14	-0.08	-0.11	-0.04	-0.03	-0.03	-0.02	-0.02	-0.02	0.28	0.19
81	Hs.81564	NM_002619	PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-0.10	-0.08	-0.09	-0.05	-0.02	-0.04	-0.06	-0.05	-0.05	0.42	0.59
82	Hs.252820	NM_002632	PGF	Placental growth factor, vascular endothelial growth factor-related protein	-0.14	-0.09	-0.11	-0.07	-0.04	-0.06	-0.06	-0.03	-0.05	0.48	0.42

83	Hs.18858	NM_003706	PLA2G4C	Phospholipase A2, group IVC (cytosolic, calcium-independent)	-0.15	-0.11	-0.13	-0.07	-0.06	-0.06	-0.07	-0.04	-0.05	0.49	0.41
84	Hs.491582	NM_000930	PLAT	Plasminogen activator, tissue	-0.17	-0.11	-0.14	-0.09	-0.06	-0.07	-0.07	-0.05	-0.06	0.53	0.41
85	Hs.77274	NM_002658	PLAU	Plasminogen activator, urokinase	-0.14	-0.10	-0.12	-0.06	-0.05	-0.06	-0.07	-0.05	-0.06	0.46	0.47
86	Hs.143436	NM_000301	PLG	Plasminogen	-0.14	-0.09	-0.12	-0.06	-0.04	-0.05	-0.06	-0.05	-0.06	0.45	0.49
87	Hs.302085	NM_000961	PTGIS	Prostaglandin I2 (prostacyclin) synthase	-0.11	-0.08	-0.09	-0.07	-0.05	-0.06	-0.05	-0.04	-0.04	0.63	0.46
88	Hs.196384	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-0.11	-0.05	-0.08	-0.07	-0.04	-0.05	-0.05	-0.04	-0.04	0.66	0.53
89	Hs.502876	NM_004040	RHOB	Ras homolog gene family, member B	0.74	0.48	0.61	0.12	0.06	0.09	0.02	0.00	0.01	0.15	0.01
90	Hs.519842	NM_003804	RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	-0.07	-0.06	-0.07	-0.06	-0.04	-0.05	-0.06	-0.03	-0.04	0.72	0.65
91	Hs.89546	NM_000450	SELE	Selectin E (endothelial adhesion molecule 1)	-0.15	-0.10	-0.13	-0.09	-0.06	-0.08	-0.07	-0.04	-0.05	0.59	0.43
92	Hs.82848	NM_000655	SELL	Selectin L (lymphocyte adhesion molecule 1)	-0.11	-0.07	-0.09	-0.04	-0.04	-0.04	-0.05	-0.04	-0.04	0.43	0.49
93	Hs.127346	NM_003006	SELPLG	Selectin P ligand	0.05	-0.01	0.02	0.18	0.09	0.14	0.02	0.01	0.02	6.81	0.87
94	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-0.11	-0.09	-0.10	-0.04	-0.03	-0.04	0.00	-0.01	-0.01	0.36	0.06
95	Hs.443914	NM_000454	SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	-0.06	-0.06	-0.06	-0.06	-0.05	-0.06	-0.05	-0.05	-0.05	0.87	0.76
96	Hs.68061	NM_021972	SPHK1	Sphingosine kinase 1	-0.08	-0.05	-0.06	-0.06	-0.05	-0.05	-0.05	-0.03	-0.04	0.91	0.69
97	Hs.89640	NM_000459	TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	-0.04	-0.04	-0.04	0.03	0.02	0.03	-0.03	-0.03	-0.03	-0.66	0.66

98	Hs.516578	NM_006287	TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	0.55	0.31	0.43	0.37	0.22	0.30	0.31	0.17	0.24	0.70	0.57
99	Hs.438231	NM_006528	TFPI2	Tissue factor pathway inhibitor 2	-0.13	-0.10	-0.12	-0.06	-0.04	-0.05	-0.07	-0.04	-0.05	0.42	0.45
100	Hs.2030	NM_000361	THBD	Thrombomodulin	-0.03	-0.04	-0.04	-0.01	0.00	-0.01	-0.01	-0.02	-0.01	0.15	0.38
101	Hs.164226	NM_003246	THBS1	Thrombospondin 1	0.64	0.38	0.51	0.07	0.04	0.06	-0.01	-0.01	-0.01	0.11	-0.02
102	Hs.522632	NM_003254	TIMP1	TIMP metalloproteinase inhibitor 1	0.65	0.37	0.51	0.62	0.41	0.52	0.45	0.26	0.35	1.01	0.69
103	Hs.241570	NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)	-0.08	-0.06	-0.07	-0.04	-0.03	-0.04	0.00	-0.02	-0.01	0.50	0.15
104	Hs.211600	NM_006290	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	-0.02	-0.01	-0.02	0.02	-0.01	0.00	-0.01	-0.02	-0.02	-0.32	1.08
105	Hs.119684	NM_003841	TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	0.46	0.25	0.35	0.68	0.47	0.58	0.23	0.13	0.18	1.62	0.51
106	Hs.213467	NM_003840	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	-0.08	-0.06	-0.07	0.03	0.00	0.01	-0.05	-0.02	-0.04	-0.19	0.51
107	Hs.81791	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-0.10	-0.07	-0.09	-0.03	-0.04	-0.04	-0.05	-0.04	-0.04	0.43	0.52
108	Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	-0.14	-0.08	-0.11	-0.05	-0.04	-0.05	-0.05	-0.04	-0.05	0.42	0.44
109	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	-0.07	-0.06	-0.07	-0.03	-0.04	-0.03	-0.04	-0.04	-0.04	0.52	0.58
110	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	-0.08	-0.06	-0.07	-0.03	-0.03	-0.03	-0.01	-0.03	-0.02	0.41	0.28
111	Hs.109225	NM_001078	VCAM1	Vascular cell adhesion molecule 1	-0.07	-0.07	-0.07	-0.02	-0.02	-0.02	-0.01	-0.02	-0.01	0.28	0.18
112	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor	-0.06	-0.04	-0.05	0.00	-0.02	-0.01	0.00	-0.01	0.00	0.25	0.02

113	Hs.440848	NM_000552	VWF	Von Willebrand factor	1.03	1.12	1.07	1.08	1.22	1.15	1.14	1.15	1.14	1.07	1.06
114	Hs.250	NM_000379	XDH	Xanthine dehydrogenase	0.99	0.86	0.92	1.07	1.17	1.12	0.93	0.68	0.81	1.21	0.87
115	N/A	L08752	PUC18	PUC18 Plasmid DNA	0.00	-0.04	-0.02	0.04	0.01	0.02	-0.03	-0.02	-0.03	-1.16	1.27
116			Blank		-0.05	-0.03	-0.04	-0.02	-0.02	-0.02	-0.02	-0.02	-0.02	0.45	0.49
117			Blank		0.05	0.03	0.04	0.03	0.02	0.02	0.02	0.01	0.02	0.64	0.48
118	N/A	SA_00005	AS1R2	Artificial Sequence 1 Related 2 (80% identity)(48/60)	0.06	0.02	0.04	0.06	0.03	0.05	0.06	0.04	0.05	1.10	1.15
119	N/A	SA_00004	AS1R1	Artificial Sequence 1 Related 1 (90% identity)(54/60)	0.08	0.02	0.05	0.07	0.04	0.05	0.09	0.04	0.06	1.10	1.35
120	N/A	SA_00003	AS1	Artificial Sequence 1	0.12	0.09	0.11	0.13	0.10	0.12	0.09	0.10	0.09	1.10	0.86
121	Hs.544577	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1.06	1.18	1.12	1.08	1.22	1.15	1.20	1.30	1.25	1.02	1.11
122	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	0.20	0.08	0.14	0.20	0.06	0.13	0.08	0.05	0.07	0.93	0.47
123	Hs.509736	NM_007355	HSPCB	Heat shock 90kDa protein 1, beta	0.12	0.03	0.08	0.14	0.06	0.10	0.04	0.02	0.03	1.32	0.37
124	Hs.509736	NM_007355	HSPCB	Heat shock 90kDa protein 1, beta	0.16	0.10	0.13	0.20	0.12	0.16	0.09	0.03	0.06	1.27	0.49
125	Hs.520640	NM_001101	ACTB	Actin, beta	1.08	1.20	1.14	1.07	1.21	1.14	1.17	1.31	1.24	1.00	1.08
126	Hs.520640	NM_001101	ACTB	Actin, beta	1.11	1.22	1.16	1.08	1.15	1.12	1.19	1.32	1.25	0.96	1.08
127	N/A	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	1.10	1.19	1.15	1.08	1.18	1.13	1.19	1.28	1.23	0.98	1.07
128	N/A	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	1.10	1.23	1.17	1.10	1.20	1.15	1.21	1.34	1.28	0.99	1.09

Host defenses to *Rickettsia rickettsii* infection contribute to increased microvascular permeability in human cerebral endothelial cells

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INTRODUCTION

Rickettsioses are arthropod-borne diseases caused by gram-negative obligately intracellular bacteria that belong to the alpha subdivision of the proteobacteria. These diseases are still prevalent in many parts of the world and include Rocky Mountain spotted fever (RMSF, the most common rickettsiosis in the US), epidemic, and endemic typhus caused by *Rickettsia rickettsii*, *R. prowazekii*, and *R. typhi*, respectively.

The main target cell is the microvascular endothelium, leading to a disseminated infection whose most severe complications include a vasogenic cerebral edema and non-cardiogenic pulmonary edema. These two complications are assumed to be responsible for most of the morbidity and mortality seen in humans. Immunohistochemical studies performed on human tissues and animal models of rickettsioses reveal the presence of rickettsiae in the microvascular endothelium. The lungs show interstitial pneumonitis and presence of proteinaceous fluid in the alveolar spaces. In severe cases, diffuse alveolar damage occurs. Studies from brain tissues show rickettsial organisms in the microvascular endothelium, perivascular inflammation composed of mononuclear inflammatory cells and parenchymal edema. However, the mechanisms responsible for increased microvascular permeability are completely unknown.

The pro-inflammatory cytokines TNF- α and IFN- γ have been shown to be important mediators of antirickettsial immunity partly through the induction of nitric oxide in rickettsiae-infected endothelial cells(2-3,6-7,12). Depletion of these two cytokines results in a fatal overwhelming infection due to uncontrolled rickettsial proliferation. Likewise the importance of CD8⁺ T-lymphocytes *in vivo* in clearing rickettsial infections has been well documented(10). Mice

depleted of CD8⁺ T-lymphocytes experience a fatal, overwhelming infection when infected with sub-lethal doses of *R. conorii*. Taken together these results demonstrate the importance of a host inflammatory response in clearing infection. However, what has not been taken in to account is the relative influence of the host immune response on the endothelial dysregulation experienced during acute rickettsioses.

Proper endothelial barrier function is regulated by a series of protein complexes that exist at the intracellular borders between cells. In the brain, tight- and adherens-junctions form the molecular barrier that regulates the flow of solutes from the blood in to the parenchyma. Two proteins of particular importance are p120-catenin (p120) and β -catenin. These two proteins serve to regulate the functional interaction of VE-cadherin with the actin cytoskeleton thereby establishing a molecular fence at cell junctions. The ubiquitous expression of these two proteins makes them an attractive target for the study of the functional alterations that may occur during rickettsial infection.

The present study was conducted with the purpose of creating an *in vitro* model to study mechanisms of permeability across rickettsiae-infected microvascular endothelial cells derived from the human brain. Here, we report the effects of *R. rickettsii* and three important cytokines (TNF- α , IFN- γ , IL-1 β) on the permeability of human brain microvascular endothelial cell monolayers. We were able to demonstrate that: 1) increases in permeability (decreased transendothelial resistance or TER) across the endothelial monolayers were directly proportional to the rickettsial inoculum 2) the addition of specific cytokines, either in combination or alone, can cause a synergistic increase in permeability when administered with rickettsiae and 3) the

SV-HCEC cell line produces anti-rickettsial nitric oxide in response to the inflammatory stimuli responsible for increased microvascular permeability.

MATERIALS AND METHODS

Rickettsial stocks and cell lines

Rickettsia rickettsii (Sheila Smith strain) was originally obtained from Dr. David Walker and was passaged in our lab one time in fertilized chicken eggs. 10% yolk-sac stocks of *R. rickettsii* were propagated through two passages in Vero cell monolayers, grown in DMEM (Gibco 11965-092) + 10% Bovine Calf Serum (Hyclone) at 34°C and purified by Renografin density gradient centrifugation. Purified stocks were frozen in SPG (sucrose-phosphate-glutamate) buffer at -80°C. Rickettsial titers of the frozen stock were determined by plaque assay on Vero cells. Immortalized human cerebral endothelial cells (SV-HCEC) were a kind gift from Drs. Som Dasgupta and Robert Yu (Medical College of Georgia)(1). This cell line was maintained in M199 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, insulin transferrin-selenium (ITS-G, GIBCO 41400-045), and 100 ug/ml heparin (Sigma H1027). Endothelial cells were grown on rat-tail collagen I-coated plates and used between passage 15-25.

Measurements of transendothelial resistance

ECIS system (Model 1600R) and electrode arrays (8W10E) were supplied by Applied Biophysics (Troy, NY). For all experiments an amplitude of 1.0 volt was used at a frequency of 4000 Hz. Electrode arrays were coated with collagen type I (Upstate, Lake Placid, NY) and seeded with approximately 5×10^4 cells/well. The electrode arrays were then connected to the detection system and resistance was monitored until stabilization of the resistance values was

observed for at least 24 hours. The endothelial cell monolayers were then used for the different experiments described below. Resistance measurements were taken every two minutes and each measurement represents the average resistance of ten electrodes per well. Each experiment consisted of three wells per sample and each experiment was performed three times. The values are expressed as the average normalized resistance and standard deviation was determined every 6 hours.

Rickettsial infection of endothelial monolayers for ECIS

Endothelial monolayers were overlaid with the designated MOI of *R. rickettsii* in M199 + 1% FBS and the electrode arrays were then connected to the ECIS detection system. Negative control cells were treated with SPG buffer which had been used to resuspend the renografin purified rickettsiae.

Determination of cell death in cell monolayers

In order to ascertain the role of cell death in increased permeability across the endothelial monolayers death curves were performed. SV-HCEC monolayers were grown to confluence in 35mm plastic Petri dishes and infected with 1 MOI of *R. rickettsii*. At 24, 48 and 72 hours, the supernatants were aspirated and the monolayers were stained using the Live-Dead Viability Stain (Molecular Probes, Eugene, OR). Images from at least three high power fields were obtained using an FV-1000 Confocal Microscope and a 10x objective. Propidium iodide (PI) uptake by cells was used to calculate the percent of cells that were undergoing cell death. Quantification of cell death was performed by determining the PI positive pixels per live cell stain pixel. Data is expressed as average PI pixels per total pixels.

Response of endothelial monolayers to cytokines

SV-HCEC monolayers were treated with the recombinant human cytokines TNF- α , IFN- γ , and/or IL-1 β diluted in M199 + 1% FBS. Evaluation of permeability of rickettsiae-infected monolayers in the presence of cytokines was done by infecting cell monolayers with 10 MOI and the addition of the above mentioned cytokines. All experiments were stopped at 72 hours post-infection.

Nitrite Measurement

Nitrite was measured with the Greiss Assay as described previously(11). Briefly, 100ul of filtered cell-culture supernatant was mixed with an equal volume of 1% sulfanilamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid. A standard curve was generated using a nitrite standard from 100uM-0.78uM (Fluka).

Statistical analysis: Sigma Stat v.2.03 was used for statistical analysis of the results. Differences in permeability and cell death rates between cell monolayers were analyzed by performing t-tests or Mann-Whitney Rank Sum Test if indicated.

RESULTS

Effects of rickettsiae on SV-HCEC monolayers

Confluent SV-HCEC cells infected with *R. rickettsii* exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in resistance (Figures 1a and 1b). Resistance declined steadily over time after rickettsiae were internalized. At 24 hours, increases in permeability ranged from 12% at 1 MOI to 25% at 50 MOI. At 48 hours post-infection, monolayers infected with 1 MOI showed a 25% increase in permeability. During the first 24 to 48 hours increases in permeability were more pronounced in monolayers infected with 20 and 50 MOI as opposed to 1, 5 and 10 MOI.

Cell death rates in rickettsiae-infected SV-HCEC monolayers

Viability staining of rickettsiae-infected cells revealed that the initial loss in resistance in response to rickettsial infection is not dependent on cell death. Propidium iodide staining revealed that by 24 hours there was no significant difference in the number of PI-positive pixels (Figure 2) despite a significant decrease in resistance at the same time point. The 48 hour time point revealed a slightly higher PI positive pixel intensity compared to controls. By 72 hours the number of PI pixel intensity actually decreased, most likely due to release of the dead or dying cells from the culture dish.

Effects of IL-1 β and TNF- α on non-infected SV-HCEC monolayers

The effects of IL-1 β on SV-HCEC monolayers were broad and although clearly caused a loss of resistance, did not behave in a truly linear fashion. At all concentrations of IL-1 β , a rapid decline was observed at 10 hours resulting in roughly a 20% loss of resistance compared to untreated controls (Figure 3a and 3b). A low dose of 0.1 ng/ml demonstrated a steady recovery from the

initial loss reaching normal levels by 72 hours. However, monolayers treated with higher doses showed a steady increase in permeability which reached values between 30-50% at concentrations of 1 ng/ml to 1,000 ng/ml, respectively by 72 hours.

The effects of TNF- α on non-infected SV-HCEC monolayers were more dramatic and behaved in a dose-dependent manner (Figure 3c and 3d). Low doses of TNF- α (0.1 and 1ng/ml) showed no significant loss of resistance compared to controls. Higher doses showed a steady increase in permeability over the 72 hour time course with the highest doses having the greatest effect. The highest dose, 1000 ng/ml, demonstrated to be rapidly cytotoxic and was excluded from future experiments with rickettsiae.

Effects of IL-1 β and TNF- α on rickettsia-infected SV-HCEC monolayers

IL-1 β did not appear to demonstrate any additional impact on resistance compared to rickettsiae alone (Figure 4a). There was no significant difference in resistance between normally infected cells and those treated with additional IL-1 β until 72 hours after infection and stimulation. At this time the lower doses of cytokines actually appeared to have a greater impact on resistance than the higher doses.

The addition of TNF- α to rickettsiae-infected cells appeared to produce a synergistic decrease in resistance at 10 or 100ng/ml as early as 24 hours after stimulation (Figure 4b). The average drop in resistance in infected cells stimulated with 100ng/ml of TNF- α was greater than either normally infected cells or uninfected cells treated with the same dose of cytokine. This highest

dose was also the only dose to demonstrate a sustained decrease in resistance below that of normally infected cells.

Effect of cytokine combinations on rickettsiae-infected SV-HCEC monolayers

The addition of TNF- α , IL-1 β and IFN- γ in combinations at a dose of 0.1ng/ml had no additional impact on resistance compared to rickettsiae alone (Figure 5). However, the addition of these cytokines at 1ng/ml each began to have a more noticeable effect. The addition of all three cytokines at one time produced the greatest loss of resistance and was significantly greater than rickettsiae alone. We also observed that TNF- α and IFN- γ in combination was sufficient to produce an increased loss of resistance. However, the combination of IL-1 β and IFN- γ at 1ng/ml did not significantly affect the resistance compared to rickettsiae-infected cell alone.

Effect of cytokine stimulation on nitric oxide production

SV-HCEC infected with *R. rickettsii* and stimulated with 10ng/ml of TNF- α , IL-1 β , and IFN- γ produced high levels of nitric oxide (NO) as measured by nitrite in the supernatant (Figure 6).

We also observed that *R. rickettsii* infection alone was sufficient to induce NO production although the addition of the cytokines produced a more robust response. The closely related organism, *R. conorii*, did not produce this same effect as we saw that *R. conorii* infection alone was not sufficient to produce significant levels of NO.

DISCUSSION

The role of the host immune response in the disease pathogenesis of acute rickettsioses is a phenomenon that has not been extensively studied. Clearly, an effective immune response is critical to the effective clearance of rickettsial organisms from the vasculature. If the organisms are allowed to persist, they will eventually overtake the host resulting in severe clinical manifestations such as cerebral and pulmonary edema, eventually leading to hypovolemic shock and death. This fact is further emphasized by the available clinical data demonstrating the effectiveness of certain antibiotics at reversing the course of disease when administered early. The immune system has also developed elaborate defense mechanisms to ward off intracellular pathogens, in particular inducible Nitric Oxide Synthase (iNOS). The iNOS enzyme is transcriptionally upregulated in response to TNF- α and IFN- γ in murine endothelial cells resulting in a decrease in the number viable rickettsiae. This was further demonstrated in human endothelial cells and hepatocytes although the necessary stimulus included RANTES and IL-1 β in addition to TNF- α and IFN- γ (4). Likewise, depletion of TNF- α and IFN- γ in mice infected with *R. conorii* demonstrate an increased proliferation of rickettsiae and are no longer able to control a sub-lethal infection.

We have demonstrated the ability of an immortalized human endothelial cell line to produce nitric oxide in response to inflammatory stimuli. More importantly we have demonstrated that these inflammatory stimuli, namely TNF- α , IFN- γ and IL-1 β dramatically influence the ability of endothelial monolayers to operate as a functional microvascular barrier. The presence of *R. rickettsii* appeared to exaggerate those effects while rickettsia itself was capable of producing high levels of nitric oxide relatively early after infection. The functional significance of this

observation is an area of current investigation. Previous work by our lab demonstrated that low levels of nitric oxide introduced to *R. conorii*-infected human microvascular endothelial cells through the addition of NO-donors was not only capable of limiting the number of intracellular rickettsiae, but had no effect on the integrity of the endothelial barrier. However, higher doses of NO-donors did eventually negatively affect the cell monolayer resulting in a slow loss of resistance over 4-5 days after infection and stimulation(13).

Whether NO directly plays a role in influencing intracellular borders may be inconsequential when compared to the cumulative affects of cytokine stimulation, as demonstrated in this work. However, NO has been implicated as a mediator of increased microvascular permeability in response to such stimuli as VEGF signaling, as well as in several *in vivo* conditions including brain trauma(5,8-9). Interestingly, VEGF-mediated NO production is not regulated by the inducible NOS enzyme (iNOS), but rather by the calmodulin-activated constitutively expressed endothelial NOS isoform (eNOS). The observation that *R. rickettsii* alone is sufficient to induce high levels of nitrite in the supernatant suggests a mechanism of eNOS activation. The importance of this potential activation in rickettsial pathogenesis is unknown. This is especially interesting considering the dramatic difference observed between *R. conorii* and *R. rickettsii*, two closely related spotted fever group rickettsiae. *R. conorii*, the etiological agent of Mediterranean spotted fever, has been utilized in mice as a model of human Rocky Mountain spotted fever, caused by *R. rickettsii*. RMSF is a very severe disease with a mortality rate approaching 30% in the absence of antibiotic treatment. Mediterranean spotted fever, however, typically only reaches about a 2.5% mortality rate. The reasons for this increased virulence are

not known at this time and may potentially be dependent on differential host responses to the organisms.

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Figure 1

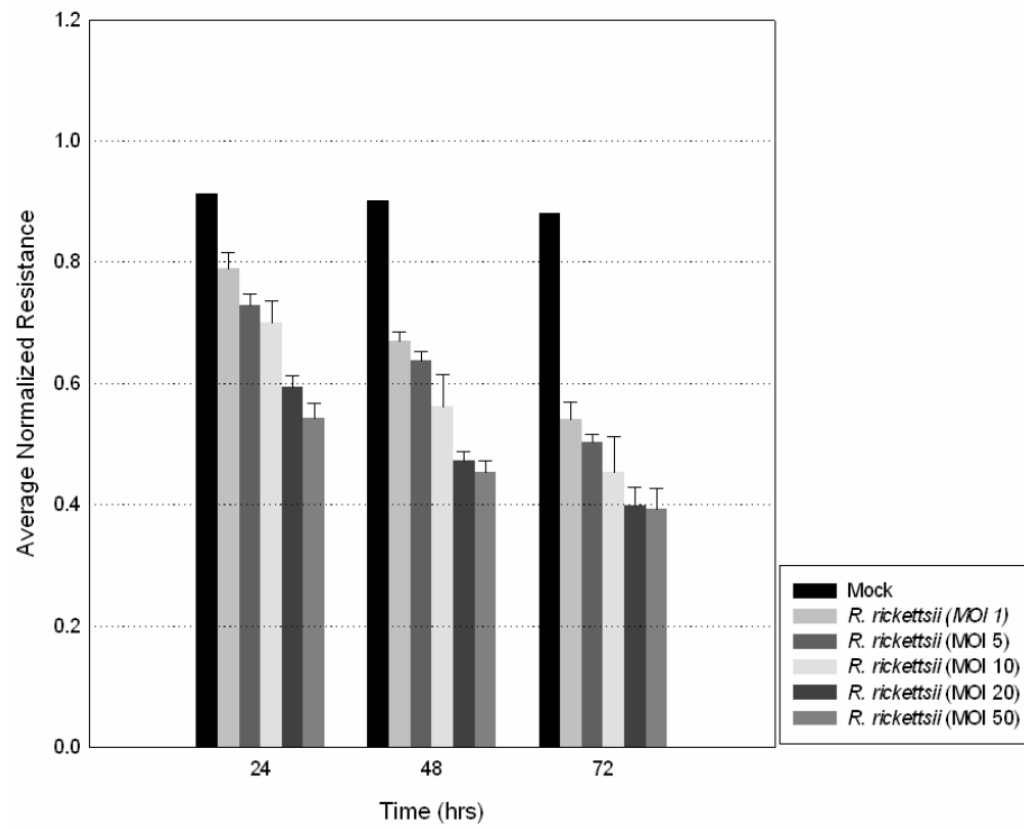


Figure 2

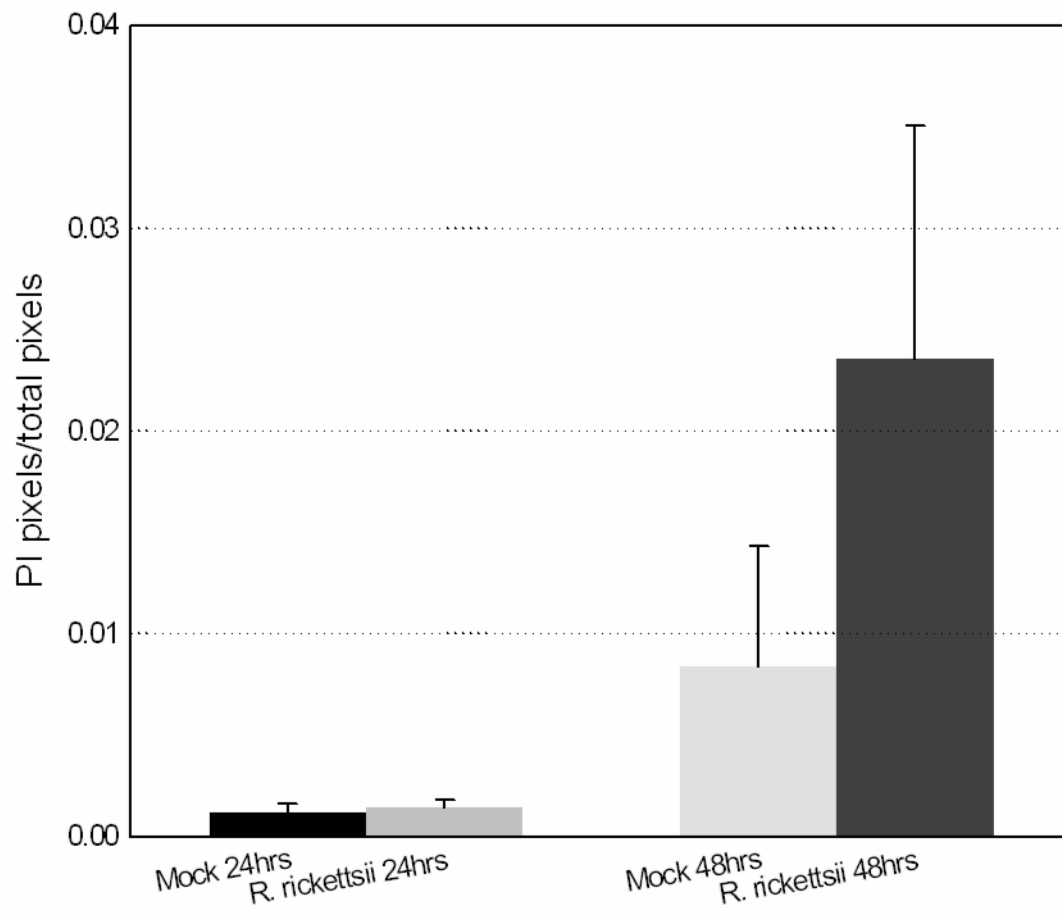


Figure 3A-D

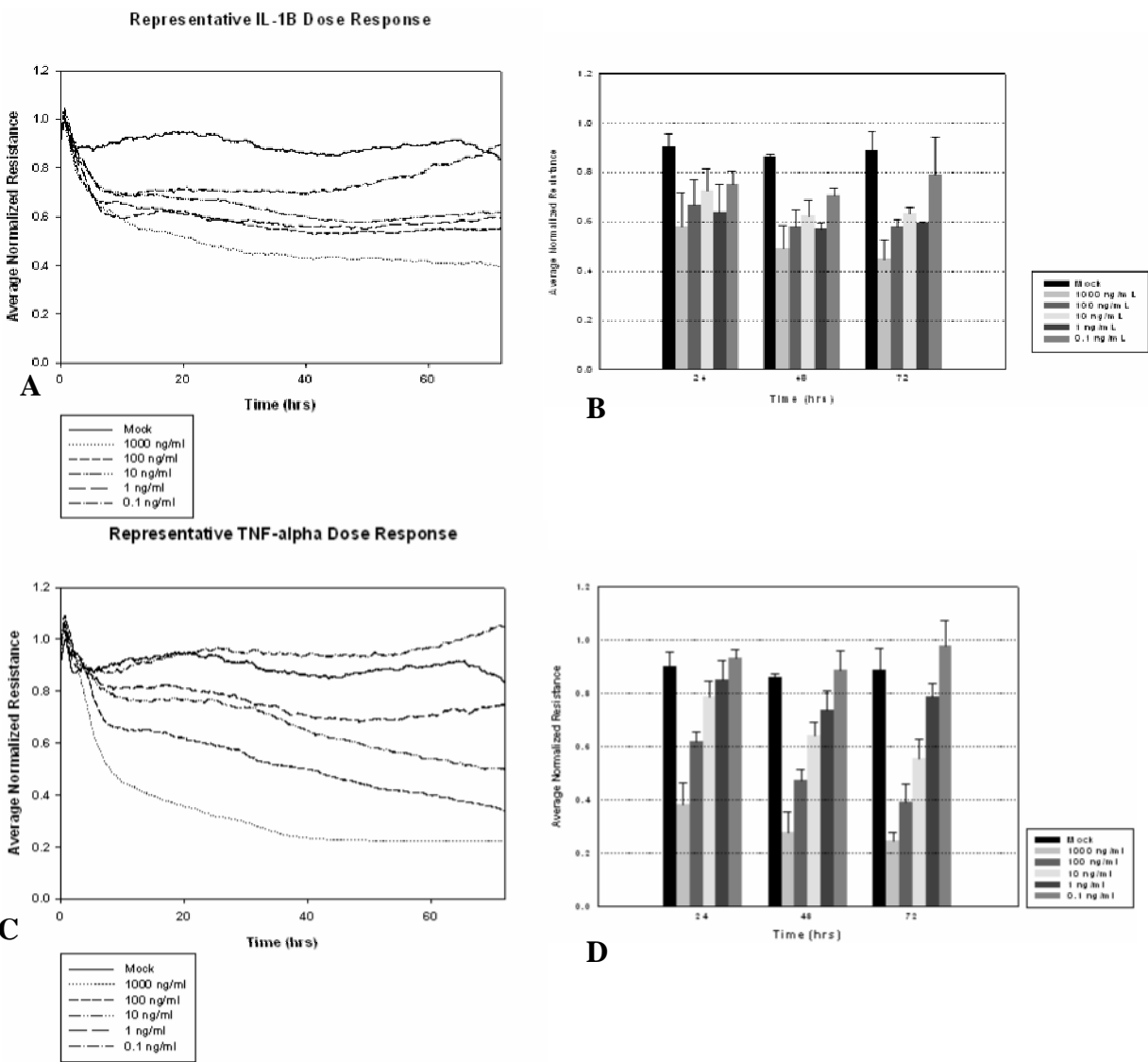


Figure 4A-B

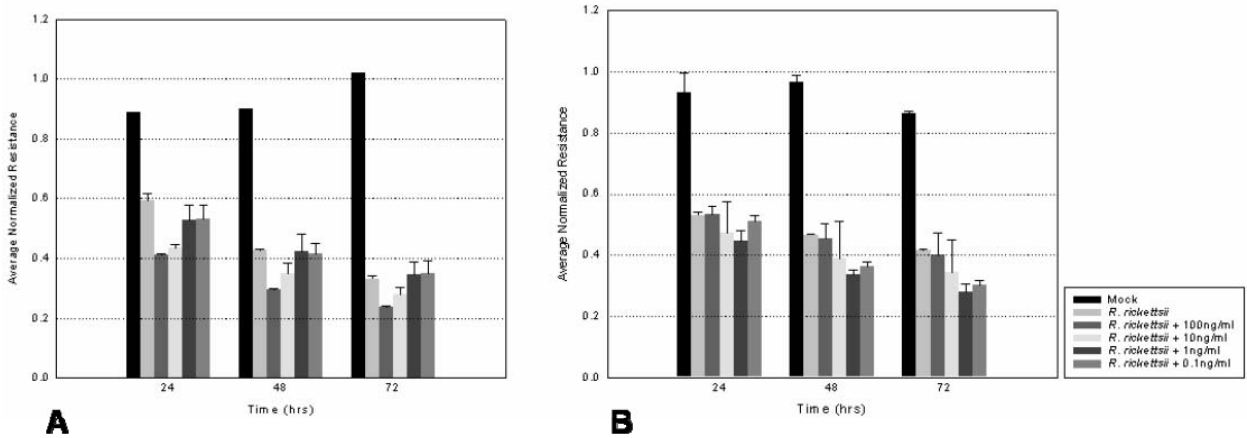


Figure 5

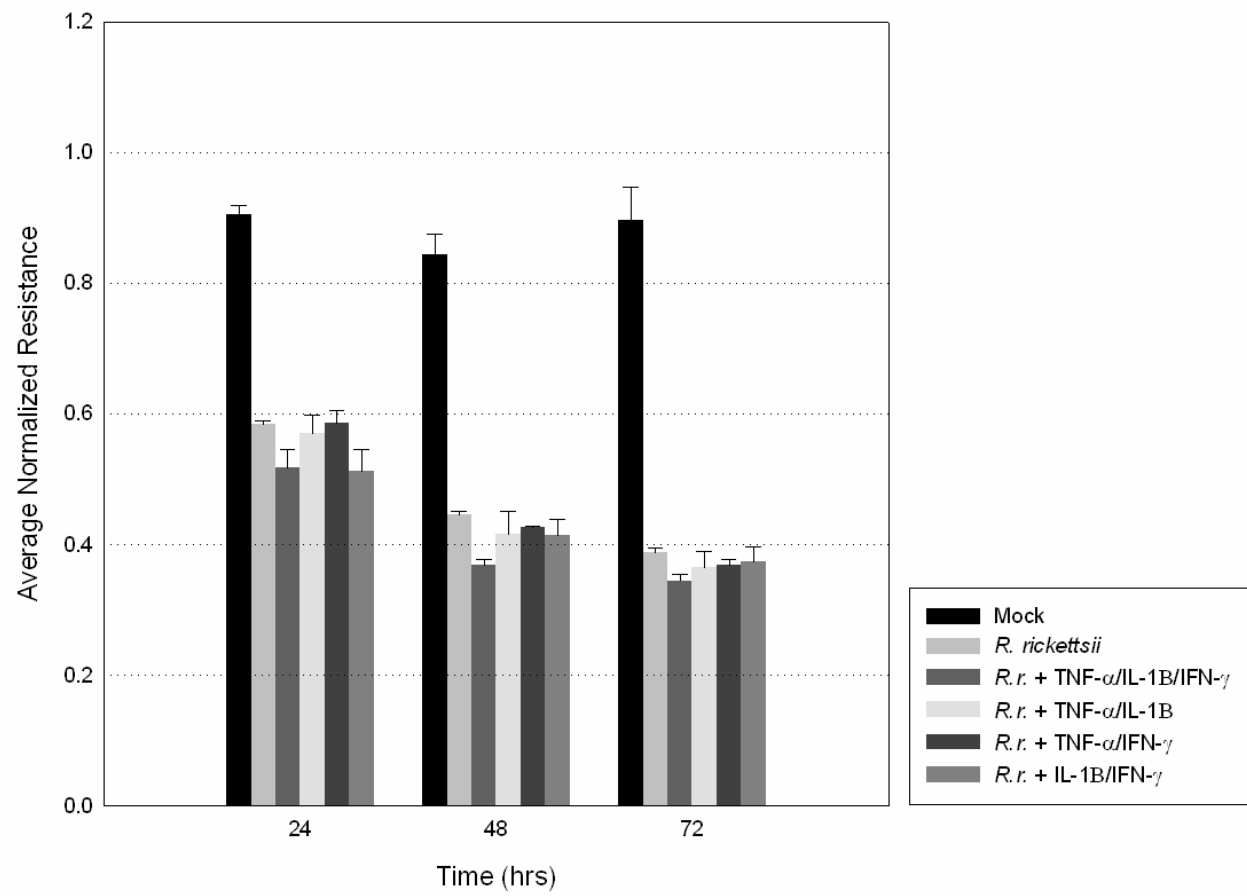
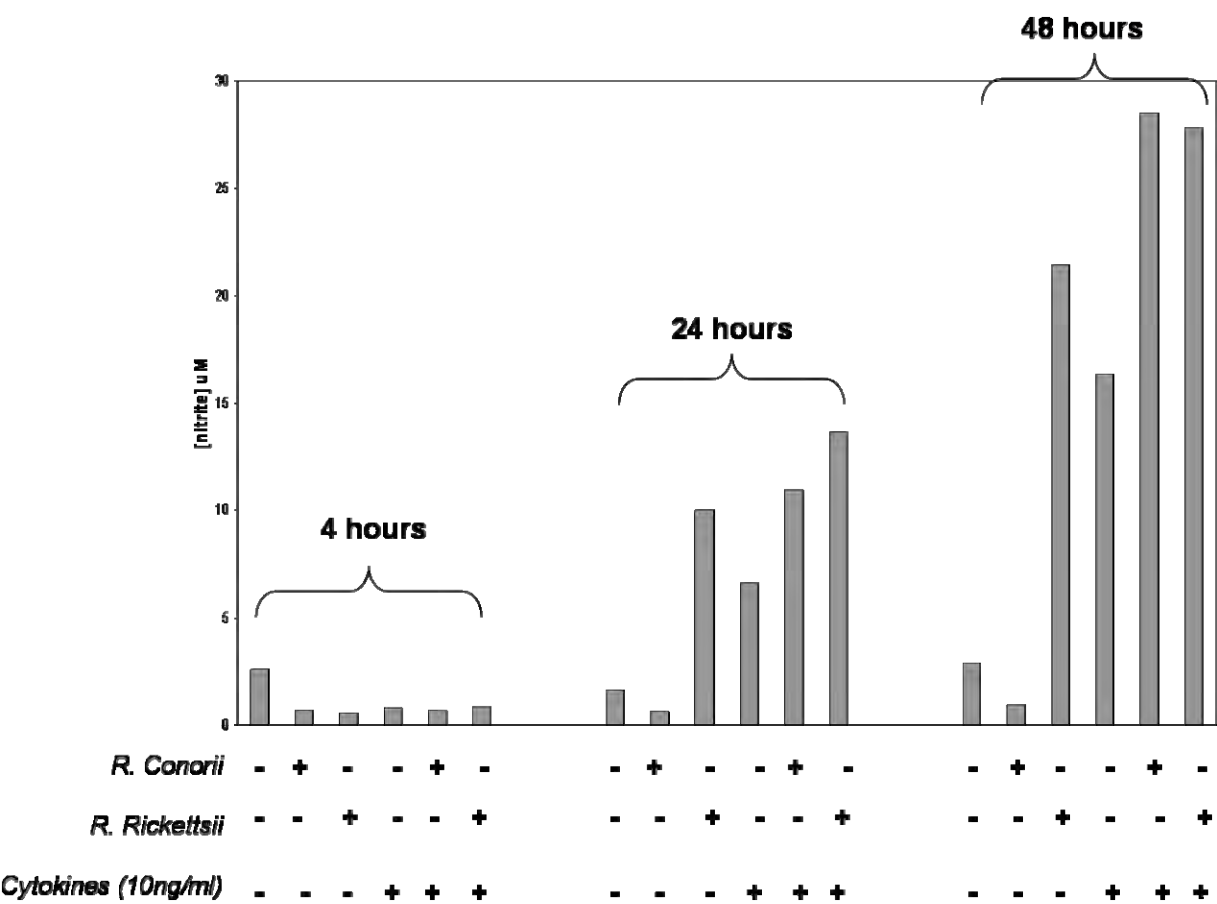


Figure 6



Nitric Oxide as a Mediator of Increased Microvascular Permeability during Acute Rickettsioses

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ABSTRACT: Rickettsiae primarily infect the microvascular endothelium, leading to changes in microvascular permeability that result in potentially severe pulmonary and cerebral edema. The mechanisms responsible for these changes are not well understood. One potential mechanism of increased vascular permeability is the anti-rickettsial nitric oxide response described by Walker and colleagues. We hypothesized that anti-rickettsial levels of nitric oxide adversely affects microvascular permeability *in vitro*. To this end we sought to describe the effects of exogenous nitric oxide on the proliferation of intracellular rickettsiae while monitoring the transendothelial electrical resistance as a measure of endothelial barrier integrity. It was determined that the addition of the NO-donor DETA NONOate at certain levels results in a dose-dependent change in electrical resistance across the monolayer while effectively limiting the number of intracellular rickettsiae in human microvascular endothelial cells. The data presented support the idea that nitric oxide produced by infected endothelial cells may be contributing to the changes in vascular permeability that occur during acute rickettsioses. Future experiments aim to elaborate on these results in a model that more clearly depicts the *in vivo* response as well as to describe the changes that occur with respect to interendothelial junctions.

KEYWORDS: Rickettsiae; nitric oxide; vascular permeability; transendothelial resistance; ECIS

INTRODUCTION

Rickettsial diseases represent some of the most severe bacterial infections known to man including epidemic typhus and Rocky Mountain spotted fever. These diseases, caused by *Rickettsia prowazekii* and *Rickettsia rickettsii*, respectively, are the result of their widespread involvement of the endothelial cells lining the microvasculature throughout the body. In severe cases, non-cardiogenic pulmonary edema and cerebral edema are the most feared complications as a result of interstitial pneumonitis and meningoencephalitis.¹ Changes in microvascular permeability

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have been described during rickettsiosis in a mouse model of infection, but these studies are very limited and do not provide an explanation for rickettsiae-induced permeability.²

A topic of intense investigation has been mechanisms of immunity to rickettsiae. Specifically, it has been shown that endothelial cells are capable of controlling rickettsiae through a nitric oxide-dependent mechanism. This response, a result of inducible nitric oxide synthase (iNOS) upregulation, is itself dependent on cytokine stimulation of the endothelium by certain pro-inflammatory cytokines, namely TNF- α , IFN- γ , IL-1 β , and RANTES.^{3,4} The antibacterial properties of nitric oxide (NO) have been well described, as has the critical role of NO as a mediator of vascular tone, platelet coagulation, and endothelial cell function.⁵⁻⁹ The apparent conundrum presented by this set of facts presents us with an opportunity to better understand the pathogenesis of rickettsial diseases as well as the role of the host immune response in the pathogenesis of the acute rickettsioses.

We sought to better understand the effects of nitric oxide on rickettsiae-infected endothelial cells and how these effects in turn affect the integrity of an endothelial monolayer. We hypothesized that anti-rickettsial levels of nitric oxide are sufficient to negatively affect the integrity of an endothelial monolayer *in vitro*. To this end, we monitored transendothelial electrical resistance after the addition of a nitric oxide donor to confluent, rickettsiae-infected human microvascular endothelial cells. We also examined the ability of the NO donor DETA NONOate to control proliferation of intracellular rickettsiae using real-time PCR.

MATERIALS AND METHODS

Reagents

MCDB 131 was purchased from Gibco (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Epidermal growth factor was purchased from Chemicon (Temecula, CA). Heparin, sulfanilamide, and naphthylethylenediamine were purchased from Sigma (St. Louis, MO). DETA NONOate was purchased from Cayman Chemicals (Ann Arbor, MI). iQ SYBR Green Supermix was obtained from BioRad (Hercules, CA).

Cell Culture

The human microvascular endothelial cell line, HMEC-1 (courtesy of Mr. Francisco Candal, CDC, Atlanta, GA), was cultured in MCDB131 + 10% FCS + 100 μ g/mL heparin + 10 ng/mL EGF in 5% CO₂ at 37°C. All experiments were performed between passages 20–25, and cells were fed with MCDB131 + 1% FCS.

Rickettsiae

Rickettsiae conorii (Malish 7) strain was kindly obtained from Drs. David Walker and Hui-Min Feng. A 10% yolk sac suspension was cultured in Vero cells (ATCC, Manassas, VA) twice and purified using renografin density gradient centrifugation. Rickettsiae were frozen at –80°C in sucrose-phosphate-glutamate buffer and their titer was determined by plaque assay on Vero cells.

Electric Cell-Substrate Impedance Sensing

HMEC-1 were cultured on 8W10E gold-coated electrodes connected to the 1600R ECIS System (Applied Biophysics, Troy, NY). The cells were cultured until a steady level of resistance was reached. The cells were then infected with 10 MOI of *R. conorii* (Malish 7) and treated with the nitric oxide donor DETA NONOate at 0, 100, and 500 μ M. The cells were monitored for 120 h with electrical resistance measurements being taken every 2 minutes. Experiments were performed in sets of four and standard deviation was determined at 6-h time points and plotted as a measure of statistical difference.

Real-Time PCR

HMEC-1 were cultured in a 24-well plate until confluent. The cells were then infected with 10 MOI of *R. conorii* and administered DETA NONOate at 0, 100, and 500 μ M. At 24-h time points the monolayer was washed with warm phosphate buffered saline and DNA was extracted using the Qiagen DNeasy Tissue Kit (Valencia, CA). Relative gene copy numbers were determined for the rickettsial citrate synthase gene, *gltA*, and the human gene for glyceraldehyde phosphate dehydrogenase, *gapdh*. The primers for *gltA* were as follows: forward primer CS-5 (GAGAGAAATTATATCCAAATGTTGAT) and reverse primer CS-6 (AGGGTCTTCGTGCATTCTT).¹⁰ The primers for *hgapdh* were as follows: forward primer hGAPDH-145(S) (CAATGACCCCTTCATTGACC) and reverse primer hGAPDH-250(AS) (GACAAGCTTCCCGTTCTCAG). iQ SYBR Green Supermix was utilized at the appropriate concentration with primers at 200 nM. One microliter of DNA from each sample was added to 24 microliters of Supermix cocktail. Real-time PCR was performed on an iCycler iQ Real-Time PCR Detection System using the following protocol: 2 min at 95°C followed by 40 cycles of 95°C for 15 seconds, 50°C for 30 seconds and 60°C for 30 seconds. relative copy numbers of *gltA* were determined by the $\Delta\Delta$ method.¹¹

Nitrite Determination

Nitrite levels were determined using the Greiss assay as previously described.³

RESULTS

Effect of Exogenous Nitric Oxide on Endothelial Permeability

HMEC-1 cells, cultured on 8W10E gold-coated electrodes, were infected with *R. conorii* at an MOI of 10 and demonstrated a level of integrity consistent with controls for the first 3 days of infection. After 72 h we saw a statistically significant drop in resistance as compared to uninfected controls. Conversely and perhaps most surprisingly, when the endothelial cells were infected with *R. conorii* and also treated with the nitric oxide donor DETA NONOate at 100 μ M we saw no statistically significant drop in electrical resistance over the course of the 5-day experiment as compared to uninfected controls. Future experiments are needed to determine if this trend would continue past 5 days. Finally, administration of DETA NONOate at

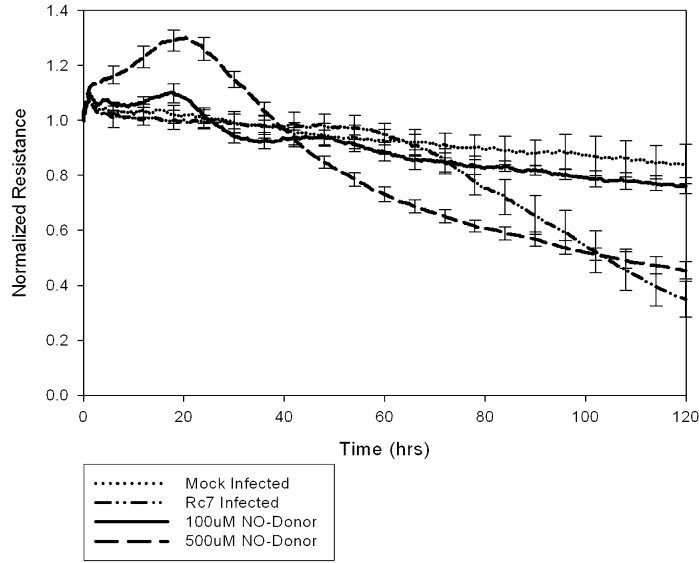


FIGURE 1. Exogenous nitric oxide influences the integrity of endothelial monolayers infected with *R. conorii*. Addition of the NO donor DETA NONOate at 100 μ M extended the life span of the infected monolayers for at least two more days. Conversely when administered at 500 μ M we saw a marked increase in electrical resistance within the first 24 h followed by a slow and steady decrease in resistance, indicating an increase in microvascular permeability. *R. conorii*-infected, non-NO-stimulated endothelial cells maintained their level of resistance for the first 3 days of infection at which time they began to undergo cell death and subsequent loss of monolayer integrity.

500 μ M demonstrated a profound effect on the integrity of the endothelial monolayer. We saw a significant increase in resistance during the first 24 h of infection and stimulation followed by a slow and steady decline in endothelial integrity as determined by a drop in resistance (FIG. 1). Taken together these findings support the hypothesis that nitric oxide can affect the integrity of rickettsiae-infected endothelial monolayers.

Effect of Exogenous Nitric Oxide on Rickettsial Gene Copy Number

HMEC-1 were cultured in 24-well plates and DNA was extracted at 24, 48, and 72 h of infection and/or stimulation with DETA NONOate. Rickettsial gene copy numbers were normalized to human *gapdh* and copy numbers of rickettsiae between samples were compared using the $\Delta\Delta$ method. We saw no significant change in the numbers of rickettsiae between cells stimulated with and without DETA NONOate for the first 48 hours. However after 72 h we saw nearly 23 times as many rickettsiae in those cells not stimulated with nitric oxide as compared to those treated at either 100 or 500 μ M (TABLE 1). Intriguingly we saw no significant difference between the ability of either dose of DETA NONOate to control rickettsiae as compared to the other.

TABLE 1. Exogenous nitric oxide limits the proliferation of intracellular *R. conorii*

	<i>R. conorii</i> / <i>R. conorii</i> + DETA NONOate (100 μ M)	<i>R. conorii</i> / <i>R. conorii</i> + DETA NONOate (500 μ M)
24 h	1.76 \pm 0.98 \times	1.15 \pm 0.17 \times
48 h	3.34 \pm 1.99 \times	2.00 \pm 0.90 \times
72 h	23.5 \pm 8.99 \times	23.45 \pm 10.54 \times

The effect is not noticed until 3 days after infection when there were significantly fewer rickettsiae as measured by real-time PCR for the rickettsial citrate synthase gene, *gltA*. Relative gene copy number was normalized to *hgapdh* and compared using the $\Delta\Delta$ method. Data indicate fold change in relative copy numbers compared to untreated, infected cells.

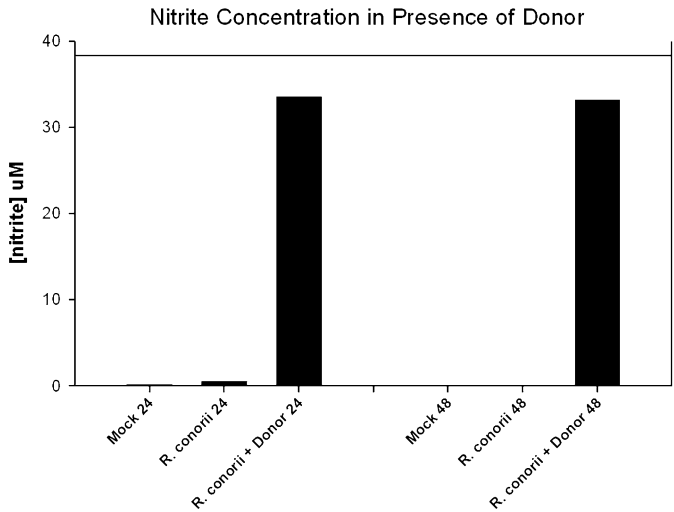


FIGURE 2. Introduction of the NO donor DETA NONOate releases nitric oxide into the environment that is subsequently converted to nitrite. Nitrite was detected within 24 h of addition of the donor molecule and remained at high levels.

***Confirmation of the Presence of Nitric Oxide By-Products
in Cell Culture Supernatants***

To confirm that we have introduced NO into the system we tested the cell culture supernatants using the Greiss reaction. We were able to detect high levels of nitrite, a by-product of NO metabolism, at 24 h after addition of DETA NONOate. The levels maintained themselves through at least the next day of the experiment (Fig. 2).

DISCUSSION

The mechanisms leading to increased microvascular permeability during rickettsial infection have not been extensively studied. There is considerable evidence supporting the role of reactive oxygen species (ROS) as a mechanism of cell damage in response to rickettsiae infection. Work by our lab suggests that the presence of ROS may have little effect on rickettsiae-induced changes in tight junction or adherens junction assembly. Instead, the accumulation of ROS may eventually lead to cell death resulting in changes in permeability at late stages of infection. To more accurately reflect the changes that take place in an *in vivo* environment we have decided to pursue a mechanism shown to be an important anti-rickettsial response and that supports the idea that increased microvascular permeability induced by rickettsiae is in part due to an immunopathological mechanism. Nitric oxide is a well known regulator of vascular tone, vascular remodeling, and interendothelial junction assembly. Previous work has also shown that both mouse and human endothelial cells are able to mount an effective anti-rickettsial response when stimulated by certain pro-inflammatory cytokines. How does this response occur in an animal and what effect will that have on the function of the microvasculature?

By introducing NO into this system of rickettsiae-infected microvascular endothelial cells we have been able to expand on the role of NO itself as not only an anti-bacterial effector molecule, but also as a potent regulator of vascular permeability. These studies have revealed that NO is very effective at limiting the number of intracellular rickettsiae. The mechanism of this inhibition however is not well understood at this time. Likewise we have shown that certain lower levels of NO donors can effectively extend the life span of endothelial monolayers and has relatively little effect on the barrier function of the monolayer. Conversely, higher levels of the NO donor produce dramatic changes in the integrity of the monolayer by first stabilizing the monolayer and then causing a slow and steady loss of barrier function. Without knowing the degree of NO release in infected endothelial cells *in vivo* at this time we are unable to make conclusions about the definite role of NO as a mechanism leading to increased microvascular permeability. The data presented do at least acknowledge the possibility that this might be the case. However, it also supports the notion that NO may purely be a defense mechanism and that there are other stimuli responsible for the loss of barrier properties. As stated previously, a true NO response by way of the iNOS enzyme is dependent on cytokine stimulation of the endothelium. It is likely that cytokine stimulation of the endothelium would cause changes in vascular permeability independent of NO production and would present a challenge in interpreting results in a system this complex, as suggested by preliminary data from our laboratory (J.P. Olano, personal communication). We expect future experiments using primary mouse brain endothelial cells to better describe the true importance of NO as not only an anti-rickettsial defense mechanism but also as a contributor to disease pathogenesis. In particular, we are interested in the effects of NO on the tight junctions that are distributed ubiquitously throughout the brain.

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[*Competing interests statement*: The authors have no competing interests.]

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Rickettsial Infections

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ABSTRACT: *Rickettsiae* are obligate intracellular α -proteobacteria that primarily target the microvascular endothelium. In the last two decades, new rickettsial pathogens have been associated with human illness around the world. Clinically, the common denominator in all rickettsioses is the development of increased microvascular permeability, leading to cerebral and non-cardiogenic pulmonary edema. With the development of powerful research tools, advances in the understanding of rickettsial pathogenesis have been dramatic. Entry into the host cell is followed by rapid escape into the cytoplasm to avoid phagolysosomal fusion. Spotted fever group rickettsiae induce actin polymerization via a group of proteins called RickA, which promote nucleation of actin monomers via the Arp2/3 complex at one rickettsial pole, propelling the bacteria across the cytoplasm and into neighboring cells. Damage to the host cell is most likely multifactorial. The most extensively studied mechanism is the generation of reactive oxygen species (ROS) and downregulation of enzymes involved in protection against oxidative injury. The significance of ROS-mediated cellular damage *in vivo* is beginning to be elucidated. The main pathogenic mechanism is increased microvascular permeability leading to profound metabolic disturbances in the extravascular compartment. The underlying factors responsible for those changes are beginning to be elucidated *in vitro* and include direct effects of intracellular rickettsiae, cytokines, and possibly activated coagulation factors—all of which most likely modify interendothelial junctions. Our knowledge on rickettsial pathogenesis will continue to expand in the near future as new research tools become available.

KEYWORDS: Rickettsiae; microvascular permeability; reactive oxygen intermediates; alpha proteobacteria

INTRODUCTION

Rickettsiae are small, obligate intracellular, gram-negative, aerobic, coccobacillary α -proteobacteria with a life cycle that includes both vertebrate and invertebrate hosts that can function both as vectors and primary reservoirs. Small mammals function as amplifying hosts and humans are merely accidental hosts. In humans, rickettsiae are responsible for systemic diseases that target the microvascular endothelium leading to highly protean clinical manifestations of which cerebral and

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pulmonary edema are the main causes of morbidity and mortality.¹⁻³ This review will focus on the interactions between rickettsiae and eukaryotic cells. I also summarize important data regarding phylogeny and virulence factors derived from the available genome sequences of selected rickettsiae.

PHYLOGENY

The alpha subdivision of the proteobacteria contains a large number of genera including plant pathogens (*Agrobacterium* and *Rhizobia*), human pathogens (*Brucella*, *Bartonella*, and *Rickettsia*), and insect endosymbionts (*Wolbachia*).⁴ Their life styles represent a wide spectrum including pericellular parasites of plants, facultative intracellular human pathogens, obligate intracellular human pathogens, and plant and insect symbionts. The transition to the intracellular life style that characterizes the *Rickettsiales* most likely occurred far back in evolutionary time as shown by phylogenetic trees that compare available sequences in the α -proteobacteria. Genome sizes in this subdivision range from 1–9 Mb. It is estimated that the ancestral proteobacterium had 3,000–5,000 genes.⁴ From this ancient ancestor, intracellular bacteria evolved with invertebrates, animals, and humans by genome reduction. In contrast, soil-growing plant-associated bacteria evolved by genome expansion. Perhaps, one of the most successful symbiotic relationships on the planet occurred approximately 2,000 million years ago when an ancient proteobacterium invaded a eukaryotic cell and eventually evolved into mitochondria.^{5,6} In fact, the closest modern relative to mitochondria is *R. prowazekii*.^{7,8} Regardless of their life styles, the α -proteobacteria are characterized by their chronic interactions with their hosts or vectors. As such, they have developed exquisite molecular mechanisms responsible for the manipulation of the host cell so that their survival is ensured.

The complete phylogeny of *Rickettsiae* is kingdom, Bacteria; phylum (VII), Proteobacteria (alpha subdivision); class (I), *Rhodospirilli*; order (II), *Rickettsiales*; family, *Rickettsiaceae*; genus, *Rickettsia*.

PATHOGENESIS

For obligate intracellular bacteria, pathogenesis is a concept that involves multiple steps: (1) transmission, (2) entry, (3) initial spread from point of entry to other organs, (4) contact with target cell (adherence and invasion), (5) survival within the host (avoidance of host defenses and adaptation to the host environment), and (6) extension of the niche (modulation of host biology, multiplication and survival). I focus on the steps that involve the interaction between rickettsiae and their eukaryotic host cell.

With the advent of complete rickettsial genome sequences, much more is known about their metabolic capabilities, pathogenesis, and potential virulence factors. However, it is worth mentioning that 49% of the open reading frames (ORFs) in *R. conorii* and 37.3% of the ORFs in *R. prowazekii* correspond to proteins of unknown function and with no homologs in databases.^{5,6,9} Simply put, there is a large

amount of work to be done in order to understand how these organisms have been so successful in nature and how they induce disease in humans.

In vitro, rickettsiae can be grown in almost any cell line available (epithelial, endothelial, fibroblastic, hematopoietic), implying that their receptor is a ubiquitous protein present in eukaryotic cells. In fact, recent evidence shows that Ku70, a protein that is present in the nucleus, cytoplasm, and lipid raft microdomains in the plasma membrane, might serve as a receptor for *R. conorii*.¹⁰ Ku70 is a multifunctional protein in eukaryotic cells whose main function in the nucleus (along with Ku80) is to serve as part of the DNA-dependent protein (serine/threonine) kinase system. In the cytoplasm, Ku70 functions as an inhibitor of Bax-mediated apoptosis, and in the plasma membrane as a fibronectin binding protein. Ubiquitination seems to be essential for Ku70 to serve as a rickettsial receptor. *In vivo*, rickettsiae target the microvascular endothelium.^{3,11,12} Minor secondary targets include cells in the medial layer of arterioles and macrophages. Internalization is active both at the target cell and the bacterium and requires active protein synthesis by the bacterium. The signaling pathways involved in rickettsial entry have been recently characterized and include recruitment of the actin-nucleating complex Arp 2/3 to the site of attachment, and the interaction of several signaling molecules including the small GTPase Cdc42, phosphoinositide 3-kinase, protein tyrosine kinases (specifically the Src family), and focal adhesion kinase (FAK).¹³ Potential adhesins have been identified by experiments performed with monoclonal antibodies and include OmpA and OmpB in spotted fever group rickettsiae and OmpB in typhus group rickettsiae.^{14–18} Once attached to the host cell, rickettsiae enter the intracellular environment by a process of induced phagocytosis by the “zipper mechanism” in which focal rearrangements of the actin cytoskeleton occur below the site of attachment leading to progressive apposition of the eukaryotic cellular membrane over the bacterium. Once rickettsiae are internalized, escape from the phagosome into the cytosolic compartment takes place after several minutes and is mediated by upregulation of genes coding for proteins with membranolytic activity, namely *tlyC* (hemolysin C) and *pld* (phospholipase D).^{19,20} Once in the cytoplasm rickettsiae are not at risk of being destroyed by lethal lysosomal enzymes and the human host has to use other mechanisms to eliminate them. This is successfully done in the majority of cases with the exception of latency in *R. prowazekii* infections and the still controversial evidence of chronic infections caused by *R. helvetica*.^{21–23}

Once in the cytoplasm, rickettsiae obtain the necessary nutrients to survive in the intracellular milieu from the host cell. Many of the genes necessary to synthesize certain metabolic products have been replaced by genes encoding for transport systems, including ATP/ADP translocases that probably entered the rickettsial genomes by horizontal transfer from *Chlamydia*.^{24–27} Due to their intracellular niche, the “tempo” of rickettsial genome evolution is much slower when compared to extracellular or facultative intracellular organisms. Therefore, horizontal transfers must have occurred more frequently in the early phases in the evolution of these organisms. Furthermore, horizontal gene transfers were probably very common in the early days of life on earth.²⁸ *Rickettsiae* are capable of producing ATP and most likely do so after depleting the host cell’s ATP pool. Such function seems to be so important that there are five copies of the ATP/ADP translocase genes.^{8,29} Other important highlights in rickettsial metabolism include the presence of glutamine transporters due to the absence of glutamine synthase, and purine and pyrimidine salvage pathways

through the host. However, synthesis of deoxyribonucleotides from ribonucleotide precursors for DNA synthesis is possible since they have the ribonucleotide reductase genes necessary for such conversion. Aminoacid synthesis is very limited and most aminoacids are taken from the host cell's cytoplasm by transporters.³⁰

The spotted fever group rickettsiae are capable of polymerizing monomeric actin filaments in the cytoplasm at one of their poles.^{31,32} The net result of this action is propulsion of rickettsiae through the cytoplasm and penetration of neighboring cells through the cell membranes. This allows rickettsiae to affect short segments of the microvasculature by invading contiguous endothelial cells without being exposed to the intravascular compartment. The molecular machinery responsible for this action has been elucidated and depends on expression of RickA, a family of proteins that acts through the Arp 2/3 complex, which in turn induces actin polymerization.^{33–36} Other examples of actin-based cytoplasmic motility include *Listeria*, *Shigella*, *Burkholderia pseudomallei*, vaccinia virus, and *Mycobacterium marinum*.^{37–41} In contrast, the absence of Rick A expression in *R. prowazekii* correlates well with the complete absence of cytoplasmic motility. *R. typhi* induces actin polymerization but the actin tails are short and “hooked” producing inefficient circular movements within the cytoplasm. Since *R. typhi* also lacks Rick A protein expression, the induction of actin polymerization must be due to other still unknown protein(s).

Rickettsiae do not secrete any exotoxins and one of the best studied mechanisms by which they can exert damage to the host cell is oxidative stress. The role of oxygen radicals has been studied extensively *in vitro* and their role *in vivo* is beginning to be elucidated.^{42–46} In addition, regulatory oxygenases also seem to play an important role in rickettsial pathogenesis as shown by upregulation of the inducible form of heme oxygenase (HO-1) in *R. rickettsii*-infected endothelial cells and the subsequent upregulation of the cyclooxygenase system.⁴⁶ The latter is responsible for increased production of prostaglandins, which in turn, might have dramatic effects on the microvasculature since these substances are well known paracrine homeostatic factors of endothelial cells and the microcirculation.

Adaptation is certainly important for rickettsial organisms since they have to shuttle between vectors (arthropods) and hosts (mammals). Needless to say, the conditions in each of these environments are very different. Five gene copies with homology to *SpoT* genes have been found in rickettsiae.^{8,29} The *spoT* gene seems to play an important role when rickettsiae move to their vectors and this same gene probably plays an important role in maintaining *R. prowazekii* and *R. typhi* viable in the feces of the louse and flea vectors, respectively, under very harsh environmental conditions.

In eukaryotic cells, rickettsiae activate NF- κ B and one of the results of this activation is the inhibition of apoptosis.^{47–49} This effect is mediated via inhibition of apical caspases-8 and -9 and by controlling intracellular levels of the bcl-2 family of proteins, specifically Bid and Bad.⁵⁰ Inhibition of apoptosis *in vitro* by intracellular rickettsiae appears logical since they are obligate intracellular organisms and therefore need the cell's nutrients to proliferate. However, *in vivo*, inhibition of apoptosis seems important in the early phases of the disease when rickettsiae start proliferating in the microvascular endothelium. Later in the disease process, when adaptive immunity is in full swing, apoptosis is in fact increased in infected endothelial cells and appears to be the result of perforin and granzyme-mediated apoptotic cell death due to cytotoxic CD8⁺ T-cells.⁵¹

Rickettsiae are one of a very selected list of human pathogens that target the microvascular endothelium exclusively. In fact, the hallmark of rickettsial infections in humans is the presence of increased microvascular permeability in all organ systems.^{2,12} The main consequence of "leaky" microvessels throughout the body is escape of intravascular fluid into the interstitial space, leading to tissue edema. Such change in vital organs such as the lungs and brain is responsible for most of the morbidity and mortality observed in human infections due to non-cardiogenic pulmonary edema and cerebral edema.^{52,53} Understanding the pathogenesis of increased microvascular permeability in rickettsial infections could lead to new avenues of treatment to prevent life-threatening complications. The movement of fluids in and out of the microvascular compartment depends on physical forces across microvessels and chemical modulation of permeability.⁵⁴ Physical forces are important in other disease states such as congestive heart failure, protein-losing nephropathies or cirrhosis. However, modulation of the microvascular permeability by chemical mediators is far more powerful than their physical counterparts. Regulation of permeability occurs mostly in precapillary arterioles, capillaries per se, and postcapillary venules, which are the very microvessels that are targeted in rickettsial infections.

Multiple mechanisms by which rickettsiae can induce microvascular permeability have been proposed and they include: (1) endothelial denudation; (2) direct effect as a result of endothelial infection by rickettsiae; and (3) paracrine and autocrine modulation by cytokines, prostaglandins, and products of the coagulation/fibrinolytic cascades. The first mechanism is self explanatory and would explain increased permeability by the mere absence of the endothelial lining in affected microvessels. In these cases, the only structure remaining in the microvessels would be the basement membrane, which would be insufficient to keep the intravascular fluids from leaking into the interstitial space. This mechanism surely happens *in vivo*, but seems to appear late in the disease process when endothelial cells are dying from the presence of massive amounts of intracellular rickettsiae or by apoptotic/necrotic cell death due to CD8+ cytotoxic T-lymphocytes. In fact, circulating endothelial cells have been demonstrated in humans during acute infections.⁵⁵

The second mechanism is beginning to be elucidated. Preliminary experiments in my laboratory have shown that monolayers composed of microvascular endothelial cell lines derived from human brain and dermis increase their permeability *in vitro* as evidenced by decreased transendothelial resistance. These changes are not explained by cell death in the monolayers during the first 24 hours of infection.⁵⁶ The molecular mechanisms responsible for these changes are currently being studied, but most likely involve signal transduction pathways affecting the cytoskeleton and ultimately junctional proteins. The role of cytokines is also being studied in my research laboratory and preliminary studies reveal that IL-1- β and TNF- α play an important role in increasing permeability across infected monolayers. IFN- γ on the contrary seems to partially counteract some of the effects of IL-1- β and TNF- α on the infected monolayers at doses of 5 and 10 ng/mL.⁵⁶ Even though coagulation abnormalities have been described *in vitro* and *in vivo* during acute rickettsioses,^{57,58} autopsy studies performed in humans have not shown disseminated intravascular coagulation and subsequent bleeding as the underlying process responsible for fatalities. However, several products of the coagulation and fibrinolytic cascade could be responsible for increases in permeability across the microcirculation. All these potential mechanisms leading to increased microvascular permeability are by no means

mutually exclusive and most likely play additive or synergistic roles *in vitro* and *in vivo*.

Due to the obligate intracellular nature of rickettsial organisms and the absence, until recently, of genetic tools to manipulate the rickettsial genomes, determination of virulence factors has been a slow process. However, due to the availability of complete genome sequences from several rickettsial organisms, the recent reports of successful transformation of rickettsiae, and the discovery of a rickettsia-associated transposon, progress in this area will certainly accelerate exponentially.⁵⁹⁻⁶¹ The definition of a virulence factor is highly controversial and a discussion of the topic is beyond the scope of this manuscript. A simple definition of a virulence factor is that of a molecule synthesized by a pathogen that is necessary (although many times not sufficient by itself) to inflict disease in a host. As discussed previously, pathogenesis involves multiple steps and therefore, molecules (proteins, glycoproteins, lipoproteins, etc.) involved in any of those steps are potential virulence factors. Therefore, several virulence factors have already been identified based on *in vivo* and *in vitro* studies and several others are considered potential virulence factors based on genome sequences. A short discussion on this topic follows, but excellent manuscripts on the topic are available.^{29,30,62}

Entry into the host cell is mediated by both OmpA and OmpB proteins, both of which are surface exposed and use of monoclonal antibodies against them reduces entry of rickettsiae into the host cells dramatically.^{14,16} The role of phospholipase D and hemolysins A and C in phagosomal escape has also been well described.^{19,63} Once in the cytoplasm, spotted fever group rickettsiae induce polar actin polymerization via RickA, allowing them to move freely in the cytoplasm and invade neighboring cells.^{34,36,64} Factors involved in intracellular survival are probably numerous and examples include InvA, a dinucleoside polyphosphate hydrolase that hydrolyzes toxic dinucleoside oligophosphates within the host cell to produce ATP and therefore favor growth and multiplication.^{65,66} An iron-associated superoxide dismutase gene ortholog, *sodB*,²⁹ was found in *R. typhi* and probably plays a role in neutralizing reactive oxygen intermediates produced in the host cell as part of their response against rickettsial invasion. The molecular machinery necessary for export of proteins outside the bacterium is a sophisticated system that involves several molecules. In rickettsiae, type IV secretion systems play this role and several *virB* orthologs have been found, including inner membrane components (VirB4 and VirB11), periplasmic components (VirB6, VirB8, VirB10), and outer membrane components (VirB3 and VirB9).^{29,30} Autotransporters also play a role in moving molecules outside the bacterial cell. As the name implies, proteins are responsible for their own secretion outside the cell. Examples include Sca1-4 and OmpB.^{8,29,67}

Rickettsiae are susceptible to few antibiotics including the tetracyclines and chloramphenicol and are highly resistant to beta-lactams and aminoglycosides. Such a narrow susceptibility spectrum is beginning to be understood. A metallo- β -lactamase has been identified in *R. typhi* as well as several penicillin-binding proteins.²⁹ In addition, multidrug transporters (efflux pumps) have also been found including *emrB*, *bcr*, and 10 other potential ABC transporters. Because of their life cycles, rickettsiae have to adapt to markedly different and sometimes harsh environmental conditions in their arthropod vectors. Such response is called the stringent response and has been studied extensively in other bacteria. Not surprisingly, rickettsiae possess in their genomes several *spoT* genes, Ndk (nucleoside diphosphokinase),

and GppA (pppGpp-5'-phosphohydrolase)—all of which play a role in down-regulating transcription and translation by increasing the concentration of GDP 3'-diphosphate and GTP 3'-diphosphate.^{29,68}

SUMMARY

Several genes in the published and deposited rickettsial genomes code for proteins with no known function and no matches with other proteins in available databases. Therefore, the potential for research in this area is enormous. With the advent of powerful imaging and molecular techniques, exciting times lay ahead. We now have the capability of genetic manipulation of rickettsiae, eukaryotic cells, and animals in order to perform powerful experiments *in vitro* and *in vivo* to elucidate both the cellular and pathophysiologic alterations leading to the morbidity and mortality associated with these diseases.

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LB522

TGF- β 1 Expression in Human Aortic Smooth Muscle Cells (VSMC) is Down-regulated when Exposed to Nicotine In Vitro for Six Days
Owano Pennycooke, Michitaka Kawata, Matthew Martens, Louise F Strande, Riva Eydelman, Charles W Hewitt, James Alexander. Surgery, Cooper University Hospital/UMDNJ-Robert Wood Johnson Medical School, Three Cooper Plaza, Suite 411, Camden, New Jersey, 08103

Objective: Previous work from this lab has shown that exposure to nicotine causes an immediate (3 hour) increase in expression of TGF- β 1. We hypothesized that chronic exposure of VSMC to free-base nicotine *in vitro* would cause sustained up regulation of TGF- β 1.

Methods: VSMC grown *in vitro* were exposed to 10^{-6} M FBN for 24 (day 1), 72 (day 3), and 144 hours (day 6). Control cells were exposed to growth media without FBN. Expression TGF- β 1 was determined by immunocytochemistry and an intensity stain index was measured quantitatively using digital image analysis.

Results: After 24 hour exposure to FBN, TGF- β 1 was down regulated when compared to control (132 ± 7.35 vs. 152 ± 5.31 , $p=.03$). After 72, and 144 hours exposure, the decrease in expression of TGF- β 1, when compared to the 24 hours, continues and is significant, (114.4 ± 6.8 vs. 132.8 ± 7.35 , $p=.05$) and (95.2 ± 3.9 vs. 132.8 ± 7.35 , $p=.004$)

Conclusion: Although exposure of VSMC's to nicotine causes an acute increase of TGF- β 1, the long-term impact is down regulation of TGF- β 1 expression. These observations imply that TGF- β 1 is only involved in the acute phase of the inflammatory response to nicotine.

LB523

HSP-70 Expression is up regulated in Human Aortic Smooth Muscle Cells (VSMC) Exposed to Nicotine In Vitro for Six Days

Owano Pennycooke, Michitaka Kawata, Matthew Martens, Louise F Strande, Riva Eydelman, Charles W Hewitt, James Alexander. Surgery, Cooper University Hospital/UMDNJ-Robert Wood Johnson Medical School, Three Cooper Plaza, Suite 411, Camden, New Jersey, 08103

Objective: We have previously shown that HSP-70 is up regulated by VSMC in response to nicotine in an acute *in vitro* model. This phenomenon correlates with a down regulation of the apoptotic pathway. We hypothesized that chronic exposure of VSMC to free-base nicotine (FBN) *in vitro*, would cause sustained up regulation of HSP-70.

Methods: VSMC grown *in vitro* were exposed to 10^{-6} M FBN for 24 (day 1), 72 (day 3), and 144 hours (day 6). Control cells were exposed to growth media without FBN. Expression of HSP-70 was determined by immunocytochemistry and an intensity stain index was measured quantitatively using digital image analysis.

Results: In VSMC, a significant increase in HSP-70 expression was observed after 24 hours exposure to FBN when compared to control (13.6 ± 2.6 vs. 6.4 ± 1.6 $p=.02$). This increased expression continued at 72 hours compared to 24 hours (108.1 ± 3.9 vs. 13.6 ± 1.6 $p=1.9^{-7}$). However, the expression of HSP-70 did not show any significant increase from 72 hours to 144 hours (108.1 ± 3.9 vs. 110.0 ± 4.6 $p=.7$).

Conclusion: Chronic exposure to an inflammatory stimulus (nicotine) causes a progressively significant increase of HSP-70. These observations imply a mechanism whereby HSP-70 is involved in the hyperplasia and hypertrophy of VSMC after chronic exposure to nicotine.

LB524

Development of aneurysm-like remodeling on vessels subjected to impinging flow

Hui Meng, D D Swartz, Z J Wang, L Gao, Y Hoi, J Kolega, E Metaxa, M P Szymanski, A M Paciorek, J Yamamoto, E Sauvageau, E I Levy, L N Hopkins. Toshiba Stroke Research Center, State University of New York at Buffalo, 445 BRB, 3435 Main St., Buffalo, NY, 14214

Cerebral aneurysms are associated with hemodynamic stresses at vessel bifurcations, but little is known about how the hemodynamic micro-environment at such locations leads to vascular change. We hypothesize that impinging flow creates complex local hemodynamics that lead to remodeling of the wall and aneurysms occur when this remodeling goes awry.

To uncover relationships between hemodynamics at bifurcations and specific vascular changes leading to aneurysms, Y-bifurcations were created by anastomosis of carotid arteries in dogs. We determined detailed local hemodynamics through *in vivo* imaging and Computational Fluid Dynamics (CFD), then examined tissue responses

by histology and immunostaining. CFD results were superimposed on the histology. This revealed large gradients of wall shear stress and development of an intimal pad at the flow impingement site. Adjacent to the impingement, shear stress was higher but gradients lower, and a groove, indicative of early aneurysm, formed in the vessel wall. The intimal pad showed extensive intimal hyperplasia at 2 weeks but matured at 2 months, with a thicker media and resemblance to a normal bifurcation apex. The groove was striking at 2 months, with a denuded endothelial layer, disrupted internal elastic lamina, and thinned media. Immunostaining showed decreased smooth muscle cell density and proliferation and decreased fibronectin in the groove, further indicating the groove may be an early aneurysm.

This model system allows identification of hemodynamic parameters that are important for cerebral aneurysm development and exploration of vascular remodeling – healthy or pathological – in response to changing hemodynamics in a living system.

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VIRAL PATHOLOGY

LB525

Peroxyntirite-mediated damage during rickettsial infection of human microvascular endothelial cells

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The objective of this study was to determine the effects of nitric oxide and its by-products on rickettsiae-infected human endothelial cells. The HMEC-1 cell line was infected with *Rickettsia conorii* and treated with or without the nitric oxide donor, DETA NONoate and the peroxyntirite decomposition catalyst FeTMPyP. Electrical resistance of the endothelial monolayers was monitored by Electric Cell-substrate Impedance Sensing as a measurement of paracellular permeability. Rickettsial viability was measured by quantitative real-time PCR and host cell viability was measured using propidium iodide staining. The results show that high levels (500uM) of the nitric oxide donor result in a rapid decrease in electrical resistance after the first day of infection and treatment. Lower levels (100uM) however proved to delay the onset of rickettsiae-induced cell death without causing a significant loss of monolayer integrity. Interestingly, both doses of the nitric oxide donor were equally effective at decreasing the number of viable intracellular rickettsiae. Additionally, we noticed that cell death did not correlate with the decrease in electrical resistance across the monolayers. Finally, we observed that the addition of FeTMPyP appeared to reverse the effects of high levels of the nitric oxide donor and the monolayers appeared to behave more similar to non-treated, infected cells. We have demonstrated that exogenously added nitric oxide has the ability to negatively impact the integrity of the microvascular endothelium most probably through a peroxyntirite-dependent mechanism.

LB526

Suppression of lymphocyte egress in simian immunodeficiency virus-infected rhesus macaques

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Lymphocyte egress from organized lymphoid tissues is dependent on the G protein-coupled, sphingosine 1-phosphate receptor 1 (S1P₁). Together with its cognate ligand, sphingosine 1-phosphate, S1P₁ is believed to mediate cellular migration through the modulation of its expression on lymphocytes and endothelial cells within the lymphatic sinuses. Here we report correlative evidence, through the quantitation of S1P₁ protein and RNA levels, that lymphocyte sequestration is intrinsic to the development of lymphoid hyperplasia in simian immunodeficiency virus-infected macaques. Furthermore, we suggest that the spatial expression of S1P₁ contributes to the heterogeneity of viral dissemination and pathology that is characteristic of simian AIDS.

Methods utilized in this study include: 1) whole slide imaging, 2) laser-dissection microscopy, 3) *in situ* hybridization.

This research was supported by the J. David Gladstone Institutes.

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Late Breaking Abstracts

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#45

Expression of a rickettsial homologue of mitochondrial processing peptidase

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Genomic analysis revealed that rickettsiae possess gene homologues to that for mitochondrial processing peptidase (MPP). Recombinant *Escherichia coli* expressing this rickettsial protein, which was named MPP-like protein (MLP), was generated. This recombinant protein with His-tag was purified to be a single band on SDS-polyacrylamide gel (electrophoresis). Mass-spectrometry of the digests of various synthetic peptides was performed to show that the recombinant MLP possesses a peptidase activity as a monomer, although the cleavage sites in synthetic peptides were different from those by MPP (Funatsu, T. et al. Seikagaku 72(8):773,2000. in Japanese). Expression of mRNA for MLP and the protein in rickettsiae was confirmed by RT-PCR and Western blotting. Partial fractionation revealed that MLP is present in envelope fraction as well as in cytoplasmic fraction. These results might contribute to the study on the molecular evolution of MPP and MLP as well as to that on mitochondria and rickettsiae.



#46

Analysis of microvascular leakage in an *in vitro* model of the brain endothelial barrier by electric cell-substrate impedance sensing (ECIS).

Paul Koo and Juan P. Olano

University of Texas Medical Branch, Galveston, TX.



Increased microvascular permeability leading to cerebral and pulmonary edema is responsible for the morbidity and mortality seen in rickettsioses. The pathogenesis of increased microvascular permeability is unknown. We are in the process of developing an *in vitro* model of the brain endothelial barrier to study the molecular events leading to vascular leakage in acute rickettsioses. ECIS is a novel method to study transendothelial resistance (TER) across cell monolayers in real time on a continuous basis. A rat-derived microvascular endothelial cell line (RBE4) as well as human-derived primary cerebral endothelial cells (HBMEC) were grown on disposable arrays with gold electrodes. The monolayers were infected with *Rickettsia rickettsii*. Cytokines (IL-1- α , TNF- α and IFN- γ) were added to some of the infected and non-infected monolayers. Controls included non-infected monolayers without cytokine stimulation and array wells containing medium alone. Our experiments demonstrated that: a) Decreased TER (increased vascular permeability) was observed after 60 – 70 hours (RBE4) or 75 – 80 (HBMEC) of infection; b) The decrease in TER was directly proportional to the rickettsial inoculum; and c) The presence of cytokines potentiated a marked decrease in TER in the monolayers infected with rickettsiae. This *in vitro* model appears promising for the study of microvascular leakage in rickettsioses and other infectious diseases in general.



#67

Development of combinatorial peptide based libraries (adaptein) with rickettsicidal activity: Preliminary results

Paul Koo, Stanley Watowich, Robert Davey, Juan P. Olano*
University of Texas Medical Branch



Combinatorial peptide libraries (adaptein libraries) are a novel and powerful technique used to screen for compounds with potential microbicidal activity. Our system is based on delivery of genes within pantropic retroviruses coding for a scaffold protein (Venezuelan equine encephalitis virus capsid protein sub-domain fused to a peptide taken from the "tat" protein of HIV) into which small random peptide sequences (combinatorial 6-mer) are presented on an exposed loop (adaptein). The diversity of each library ranges between 2×10^7 and 2×10^8 . Typical retroviral titers are approximately 10^7 pfu/ml. A rat brain derived endothelial cell line (RBE4) was infected with the retroviral vectors containing adaptein libraries and subsequently challenged with *Rickettsia rickettsii* at a MOI of 25. At 96 hours after the challenge, surviving cells still attached to the substratum were trypsinized, replated, and allowed to grow for 48 – 72 hours before being re-challenged with *R. rickettsii* (MOI = 15). Three more rounds of re-challenges (MOI = 10) were carried out. At the end of 5 rounds of challenges, 7 foci of putative rickettsia-resistant RBE4 cells were recovered. We are currently in the process of characterizing these cells at the molecular level to elucidate the mechanisms of resistance to rickettsial challenge.



#68

Effect of *Bartonella bacilliformis* cell fractions on human vascular endothelial cell growth

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Division of Biological Sciences, The University of Montana.

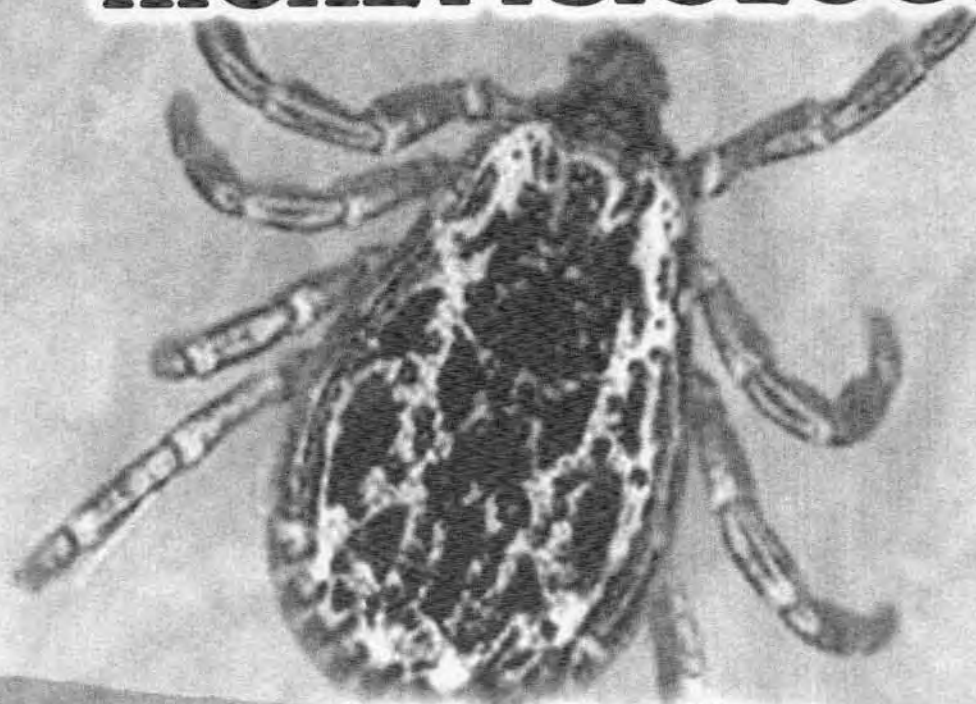


Bartonella infections can result in angioproliferative lesions of the vasculature that are potentially life threatening to humans. To investigate the proliferative response accompanying bartonellosis, cultured human umbilical vein endothelial cells (HUVECs) were exposed to various *B. bacilliformis* cell fractions. Results of this study show that the pathogen produces a proteinaceous mitogen that acts in a dose-dependent fashion *in vitro* with maximal activity at ≥ 72 h exposure, and results in a 6 to 20-fold increase in cell numbers relative to controls. The mitogen increases BrdU incorporation into HUVECs by almost 2-fold relative to controls. The mitogen is sensitive to heat or trypsin but is not affected by the LPS inhibitor, polymyxin B. The mitogen does not affect caspase 3 activity in HUVECs undergoing serum starvation-induced apoptosis. The *Bartonella* mitogen was found in bacterial culture supernatants, the soluble cell lysate fraction, and to a lesser degree in insoluble cell fractions of the bacterium. In contrast, soluble cell lysate fractions from closely-related *B. henselae*, although possessing significant mitogenicity for HUVECs, resulted in only about a two-fold increase in cell numbers. These data suggest that *Bartonella* directly affects the growth of vascular tissue, in addition to inducing angiogenic cytokines and growth factors as previously published. The mitogen is described in a second abstract.





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**DEVELOPMENT OF COMBINATORIAL PEPTIDE-BASED LIBRARIES
(ADAPTEINS) WITH RICKETTSICIDAL ACTIVITY:
PRELIMINARY RESULTS**

Olano Juan P, Koo P, and Walker DH

University of Texas Medical Branch, Galveston, TX

BACKGROUND/PURPOSE: Combinatorial peptide libraries (adaptein libraries) are a novel and powerful technique used to screen for compounds with potential microbicidal activity. Our system is based on initial delivery of small random peptide sequences (combinatorial 6-, 12-, and 18-mers, also known as adapteins) attached to the C-terminus of EGFP constructed within a replication-incompetent murine leukemia retrovirus that was co-transfected with pGag-Pol and pVSV-env into 293 cells. The diversity of the resultant recombinant retrovirus library (released into the medium) ranges between 2×10^7 and $>3 \times 10^8$. Typical retroviral titers are approximately 10^7 pfu/ml.

METHODS: A rat brain-derived endothelial cell line (RBE4) was infected with the retroviral vectors containing adaptein libraries and subsequently challenged with *Rickettsia rickettsii* at a MOI of 25. At 96 hours after the challenge, surviving cells still attached to the substratum were trypsinized, re-plated, and allowed to grow for 48–72 hours before being re-challenged with *R. rickettsii* (MOI = 15). Three more rounds of re-challenges (MOI = 10) were carried out.

RESULTS AND CONCLUSION: At the end of five rounds of challenges, several foci of putative rickettsia-resistant RBE4 cells were recovered. However, none of the surviving cells were EGFP positive. The reason for the lack of EGFP expression in these putative rickettsia-resistant cells is currently under investigation. The experiment was repeated once more exactly as described and yielded several foci of putative-resistant cells that were EGFP positive. We are currently in the process of characterizing these cells at the molecular level to elucidate the mechanisms of resistance to rickettsial challenge.

EVALUATION OF ENDOTHELIAL PERMEABILITY IN AN IN VITRO MODEL OF THE BRAIN ENDOTHELIAL BARRIER BY ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING (ECIS)

Olano JP, Koo P, and Walker DH

University of Texas Medical Branch, Galveston, TX

BACKGROUND: Increased microvascular permeability leading to cerebral and pulmonary edema is responsible for the morbidity and mortality seen in rickettsioses. For example, C3H/HeN mice infected with *R. conorii* show foci of increased vascular permeability at 72–96 hours post-infection. However, the pathogenesis of increased microvascular permeability is unknown. We are in the process of developing an in vitro model of the brain endothelial barrier to study the molecular events leading to vascular leakage in acute rickettsioses. ECIS is a novel method to study transendothelial resistance (TER) and therefore evaluate endothelial permeability across cell monolayers in real time on a continuous basis.

METHODS: A rat-derived microvascular endothelial cell line (RBE4) as well as human-derived primary cerebral endothelial cells (HBMEC) were grown on disposable arrays with gold electrodes. The monolayers were infected with 10 MOI of *Rickettsia rickettsii*. After rickettsial internalization, cytokines (IL-1- β , TNF- α , and IFN- γ), either singularly or in combination, were added at low (5 ng/ml) and high doses (20 ng/ml) to some of the infected and non-infected monolayers. Controls included non-infected monolayers without cytokine stimulation and array wells containing medium alone. Cell monolayers were monitored continuously for 96–120 hours.

RESULTS AND CONCLUSION: Our experiments demonstrated the following: (a) Decreased TER (increased vascular permeability) was observed after 60–70 hours (RBE4) or 75–80 (HBMEC) of infection. (b) The decrease in TER was directly proportional to the rickettsial inoculum. (c) In general, the presence of cytokines potentiated an earlier and more dramatic decrease in TER in the monolayers infected with rickettsiae. Synergistic effects between cytokines were documented when more than one cytokine was added to the medium. (d) Low-dose IFN- γ by itself favored cell survival as indicated by the preservation of micromotion for a longer period of time after infection. This in vitro model appears promising for the study of microvascular leakage in rickettsioses and other infectious diseases in general.

Military Health Research Forum

Foreword

Combat Casualty Care Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Organizational	Speaker/Poster Presenter	Poster Assignment Number
Isaacs	Helen	Thrombolytic and Gray Matter Structural Changes Following Moderate Traumatic Brain Injury in Rats	Albany University School of Medicine	Not Presenting	
Crusby	John	Efficacy of Countermeasures against Traumatic Brain Injuries Sustained in Airborne Operations	U.S. Army Aeromedical Research Laboratory, T.R.U.C. Research Foundation, San Antonio, TX	Poster Presenter Joe McIntire	C8
Doehring	Patricia	Serial Assessment of Mild Head Injury: Early Prediction of Outcome	University of Maryland School of Medicine, National Study Center for Trauma and EMS	Poster Presenter	C9
Friedman	Joel	A New Noncovalent Polyethylene Glycol Hb Conjugate (CTP-PEG(10)-Hb) High Viscosity, High Viscosity Pressure Blood Substitute	Albert Einstein College of Medicine of Yeshiva University	Speaker/ Poster Presenter	C2
Friedman	Joel	Vasoreactivity of PEGylated Hemoglobin: Influence of Surface Configuration of PEG-Hb in the Vasculature of Conscious Rats	Albert Einstein College of Medicine of Yeshiva University		
Friedman	Joel	Targeted to Amino Groups of Hemoglobin: New PEG-Hb Conjugates to Evaluate the New Paradigm of Designing Blood Substitutes	Albert Einstein College of Medicine of Yeshiva University		
Friedman	Joel	Stable Powdered Formulations of Hemoglobin-Based Potential Blood Substitutes	Albert Einstein College of Medicine of Yeshiva University		
Gumpel-Hill	Andrew	Development of a Temporary Implantable Biopsy for Monitoring of Glucose and Lactate during Hemorrhage	Virginia Commonwealth University	Poster Presenter	C3
Harmon	John	A Hemostatic Care Therapy System with Potential to Heal War Wounds	Johns Hopkins University	Speaker/ Poster Presenter	C4
Hayes	Ronald	Comparison of Biomarkers at Spectrum Breakdown Products, S100 β , and Tau after Traumatic Brain Injury and Ischemia	McKnight Brain Institute of the University of Florida	Speaker/ Poster Presenter	C5
Kwarg	K. Seer	Protection against Ionizing Radiation Injury by a Benzyl Sulfone Analog	Armed Forces Radiobiology Research Institute	Speaker/ Poster Presenter	C6
Kwarg	K. Seer	Radiation Protection by Alpha-Tocopherol Succinate	Armed Forces Radiobiology Research Institute	Speaker/ Poster Presenter	C7
Mayerhoff	James	Neuroprotective Strategy against Oxidative Injury from Traumatic Brain Injury (TBI) Due to Ferric Chloride Infusion	Walter Reed Army Institute of Research	Speaker/ Poster Presenter	C9
Warden	Deborah	A Randomized Placebo-Controlled Trial of Citalopram for Anxiety Disorders following Traumatic Brain Injury	Defense and Veterans Brain Injury Center, Uniformed Services University of the Health Sciences	Poster Presenter	C10

Infectious Disease Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Coordinating Organization	Speaker/Poster Presenter	Poster Assignment Number
Adams	William	Thrombotic Thrombocytopenic Syndrome (TTP) in the Elderly: A Review	Stanford University School of Medicine	Poster Presenter	10
Ben-Shoshan	Chantal	Phenyl and Tyrosine Kinase Inhibitors for the Treatment of Chronic Myeloid Leukemia: Mechanisms of Action, Clinical Trials, and Future Prospects	University of Connecticut Health Center	Speaker/ Poster Presenter	11
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	11
Hernandez	Marlene	Progress in the Development of an Anticancer Drug: From Targeted Therapy to Personalized Medicine, the Anticancer Pathways that Regulate the Pharmacokinetics and Pharmacodynamics of the New Drug	University of California, Los Angeles	Speaker/ Poster Presenter	12
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	13
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	14
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	15
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	16
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	17
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	18
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	19
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	20
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	21
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	22
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	23
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	24
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	25
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	26
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	27
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	28
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	29
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	30
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	31
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	32
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	33
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	34
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	35
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	36
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	37
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	38
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	39
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	40
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	41
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	42
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	43
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	44
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	45
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	46
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	47
Chang	Lincoln	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	48
Ward	Thomas	Investigation of the Capacity of Hemoglobin-Based Blood Substitutes to Deliver Oxygen and Nitrogen to the Tissues	Virginia Polytechnic Institute and State University, College of Veterinary Medicine	Speaker/ Poster Presenter	49
Adams	Thomas	Development of a Novel Intracellular Targeted Drug for the Treatment of Hepatocellular Carcinoma	United Medical Research Center	Poster Presenter	50

Lung Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Coordinating Organization	Speaker/Poster Presenter	Poster Assignment Number
Higgins	Jeffrey	Longitudinal Changes in Tracheobronchovascular Inflammation in Healthy Individuals: A Review	University of Illinois	Poster Presenter	1
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	2
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	3
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	4
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	5
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	6
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	7
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	8
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	9
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	10
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	11
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	12
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	13
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	14
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	15
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	16
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	17
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	18
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	19
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	20
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	21
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	22
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	23
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	24
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	25
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	26
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	27
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	28
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	29
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	30
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	31
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	32
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	33
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	34
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	35
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	36
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	37
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	38
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	39
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	40
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	41
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	42
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	43
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	44
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	45
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	46
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	47
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	48
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	49
Farrell	Thomas	Improved Outcomes in Patients with Acute Lung Injury and Systemic Inflammation: A Review	University of Illinois	Speaker/ Poster Presenter	50

Military Operational Medicine Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Coordinating Organization	Speaker/Poster Presenter	Poster Assignment Number
Adams	William	Thrombotic Thrombocytopenic Syndrome (TTP) in the Elderly: A Review	Stanford University School of Medicine	Poster Presenter	10
Adams	William	Thrombotic Thrombocytopenic Syndrome (TTP) in the Elderly: A Review	Stanford University School of Medicine	Poster Presenter	11

Preprint	Preprint	DEAR Sleep Management System: The Sleep Health Program	Walter Reed Army Institute of Medicine	Poster Presenter	
Poster	Poster	WARR Sleep Management System II: Assessment of Performance Outcomes	Walter Reed Army Institute of Medicine	Poster Presenter	932
Poster	Poster	WARR Sleep Management System II: Pharmacologic Sleep Medication for Sustaining Cognitive Functions	Walter Reed Army Institute of Medicine	Poster Presenter	
Poster	Poster	The Mechanism and Outcome of Resilience in Post-Traumatic Stress Disorder	Northwestern University (College of Arts & Sciences)	Poster Presenter	933
Poster	Poster	The Use of the Operational Epidemiological for Assessing Functional Outcomes in the Active Duty Army	Walter Reed Army Institute of Medicine	Poster Presenter	934
Poster	Poster	William H. Hines Total Sleep Deprivation and Recovery Study on Cognitive Performance	University of California, San Diego, and San Diego Veterans Affairs Healthcare System	Poster Presenter	935
Poster	Poster	Performance Benefits of a Behavioral Remedial Program: Total Company Performance Improvement in a Chemical Warfare Unit (CWU) in 2011	Lawrence Livermore National Security and U.S. National Chemical Stockpile Disposal Center	Poster Presenter	936
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	937
Poster	Poster	The War Program: An Interdisciplinary Program to Address the Health Needs of Active Duty Military Personnel and Veterans	The University of Texas System (University of Texas at Dallas)	Poster Presenter	938
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	939
Poster	Poster	The Complex Assessment of Complex Trauma Following a Natural Disaster: A Case Study of the 2011 Tōhoku Earthquake	University of Maryland System (College of Health, Behavior, & Society)	Poster Presenter	940
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	941
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	942
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	943
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	944
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	945
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	946
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	947
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	948
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	949
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	950
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	962
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	968
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	969
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	971
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	979
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	981
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	982
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	983
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	984
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	985
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	986
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	987
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Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	999
Poster	Poster	University of Maryland System	University of Maryland System	Poster Presenter	1000

Technology Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Contracting Organization	Poster/ Speaker	Poster Assignment Number
Chen	Huang-Ping	Computer-Aided Diagnostic System for Detection of Breast Masses on Digital Mammograms	University of Michigan	Poster Presenter	T1
Quinn	Steven	Enabling Technologies for Advanced Soft Tissue Modeling	Massachusetts General Hospital	Speaker/ Poster Presenter	T4
Gould	Robert	Development of a New Medical Image Archive (MIA) for the Integration of Image Data from Multiple Institutions	University of California at San Francisco	Poster Presenter	
Hampford	Blake	Mini-Robot Design for Minimally Invasive Surgery in the Battlefield: Breaking the Size Barrier for Surgical Manipulators	University of Washington	Not Presenting	
Mayes	Jeffrey	High-Sensitivity Detection of Biological Toxins	American Registry of Pathology/ Armed Forces Institute of Pathology	Speaker/ Poster Presenter	
Kohonen	Thomas	Development of an Advanced Protein Blood Processing System	Alkermes Medical, Inc.	Poster Presenter	
Sohn	David	Remote Optimization of Image and Diagnostic Quality during Real-Time Telemedicine Echo Screening of Veterans at Risk for Cardiac Disease: A New Capability Developed in Support of Our DOD	Oregon Health & Science University	Poster Presenter	T6
Baker	Andrew	Validation of a Real-Time Heart Rate Variability	Midwest Research Institute	Poster Presenter	T7
Reilly	Brian	Portable, Noninvasive System for Use in Triage and Treatment of Shock: System Design and Characterization	University of Massachusetts Medical School	Speaker/ Poster Presenter	T8
Thompson	Kai	Novel Acoustic Arrays for Portable Ultrasound	GE Global Research	Speaker/ Poster Presenter	T9
Venky	Ivan	Advanced Soft Tissue Modeling for Telemedicine and Surgical Simulation	Children's Hospital of Los Angeles	Poster Presenter	T10
Yoshimizu	Ryota	Use of Tele-Technology for Heart Disease Management: Improving Clinical and Economic Outcomes in a Managed Care Population	Portland VA Medical Center	Poster Presenter	T11

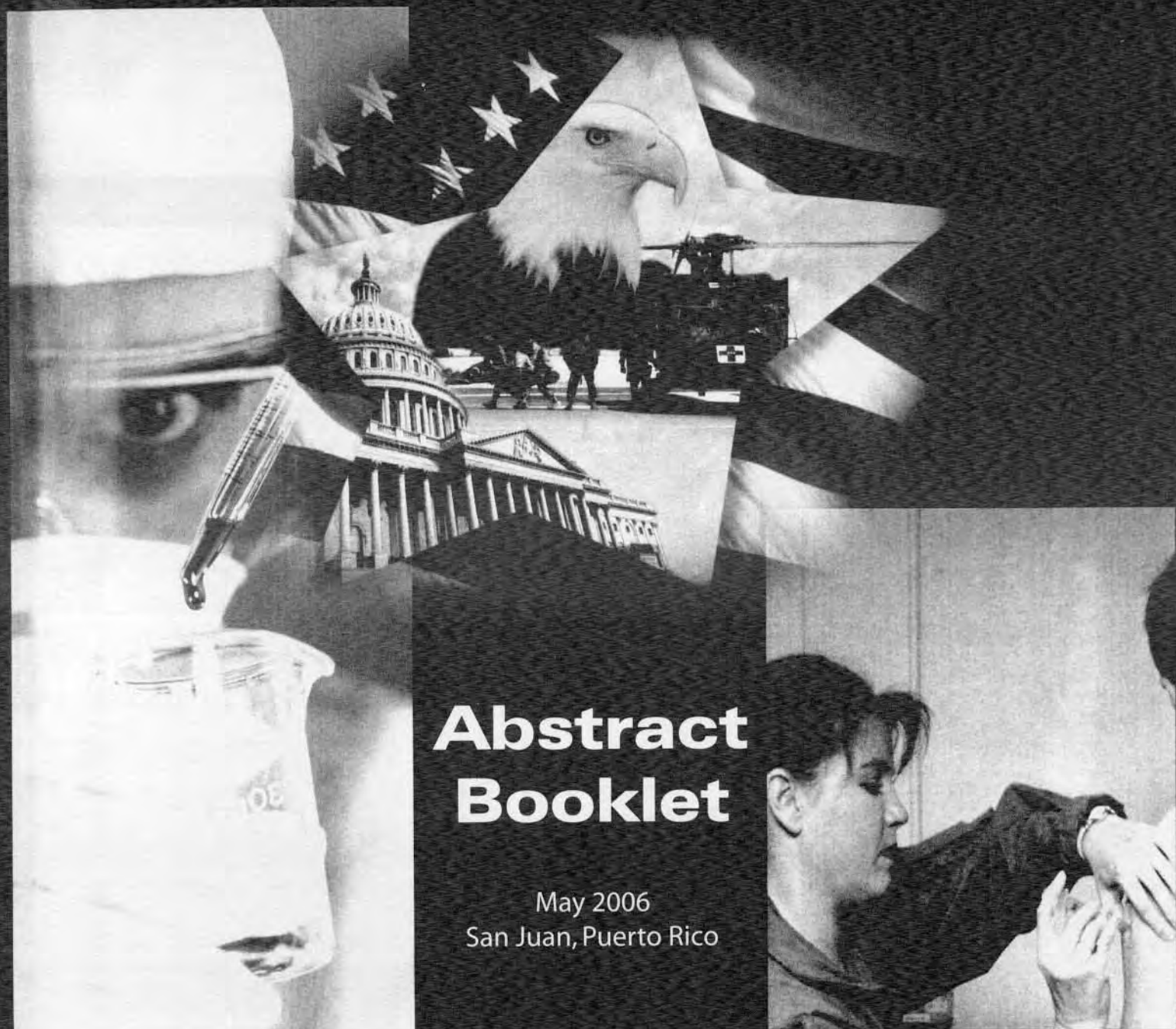
Wellness/Fitness Research

Principal Investigator Last Name	Principal Investigator First Name	Abstract Title	Contracting Organization	Poster/ Speaker	Poster Assignment Number
Chen	Andrew	Use of the F1000 Research as a Model for Publishing and Assessing Long-Term Research (Chen et al.)	University of Texas Medical Branch, Galveston	Poster Presenter	919
Chen	Andrew	The Effect of a Novel Medical Approach to Assess Health Research among U.S. Army Personnel	Naval Institute for Research and Development	Speaker/ Poster Presenter	921
Chen	Andrew	Enhancing Physical Performance of Care for Use in Military and Sports Performance Studies	Naval Institute for Research and Development	Poster Presenter	922
Chen	Andrew	Effect of Exercise on Sleep Quality in Military Personnel	Naval Institute for Research and Development	Poster Presenter	923
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	924
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	925
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	926
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	927
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	928
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	929
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	930
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	931
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	932
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	933
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	934
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	935
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	936
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	937
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	938
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Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	989
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Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	991
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	992
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	993
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	994
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	995
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	996
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	997
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	998
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	999
Chen	Andrew	Physical Activity Experience Design for Improved Performance	University of Texas Medical Branch, Galveston	Poster Presenter	1000

McMartin	Kenneth	Efficient natural Immunity to human herpes 8 infection leads to increased cancer risk	Louisiana State University Health Sciences Center	Poster Presenter	W12
Miller	Clasilia	Hispanic Serving Institutions: No Title/Abstract Submitted	University of Texas at San Antonio Health Sciences Center	Poster Presenter	W13
Morgan	Thomas	Training Administrative Responsibility in International Health Practice in the Air Force	University of New Mexico	Not Presenting	
Rivero-Cano	Alarcon	Hispanic Serving Institutions: No Title/Abstract Submitted	University of Puerto Rico	Poster Presenter	W14
Reynolds	Herbert	Population Health Trial for Smokeless Tobacco Cessation with Military Personnel	Oregon Research Institute	Poster Presenter	W15
Slip	Ann	Substance Problems, Suicidality, and Family Maladjustment: Do We Only See the Tip of the Iceberg?	State University of New York	Speaker/ Poster Presenter	W16
Sprung	William	Hispanic Serving Institutions: No Title/Abstract Submitted	University of Texas at Brownsville	Poster Presenter	W17
Trent	Linda	Tobacco Cessation Interventions for United States Marine Corps Recruits	Naval Health Research Center	Not Presenting	
Vaduz	Chao	Global Gene Expression in Preclinical Models of Alcoholism	New York University Medical Center	Poster Presenter	W18
Velez-Jones	C. Fernando	Kainic Receptors Are Very Sensitive Targets of Alcohol	University of New Mexico Health Sciences Center	Poster Presenter	W19
Wood	W. Thomas	High-Density Lipoprotein and Lipoprotein(a) Levels in Coronary Artery Disease: A Meta-Analysis of Prospective Cohort Studies of the Role of Lipoprotein Structure and the LDL Complex	University of Minnesota School of Medicine	Poster Presenter	W20
Yan	Qing-Shan	Investigation of the Role of the Tumor-Suppressing p53 in Regulation of Glutathione Peroxidase Expression in Human Liver	University of Illinois at Chicago School of Medicine	Poster Presenter	W21

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Infectious Disease Research

REPLICATION OF A CHIMERIC HEPATITIS C-DENGUE VIRUS

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BACKGROUND: Hepatitis C virus (HCV) is the major cause of posttransfusion and community-acquired chronic hepatitis. Initial steps of HCV infection, a critical determinant of tissue tropism and therefore pathogenesis, are largely hypothetical. The main cause hampering sufficient studies on HCV binding and internalization is the ongoing absence of a robust cell culture system allowing the release of mature HCV virions.

PURPOSE: To circumvent this limitation, the aim of our study was to construct and clone a chimeric flavivirus, expressing the HCV envelope proteins E1 and E2 within a dengue virus (DENV) backbone, which is a close relative to HCV and can be efficiently propagated in cell culture.

METHODS: A monocistronic chimeric cDNA clone was constructed by a PCR based method. The principal strategy for the construction of the chimeric flavivirus was replacement of DENV premembrane (prM) and E-protein with the E1-E2-p7 encoding region of HCV. Transfection of this clone, but not of a non-replicative control clone, led to production of chimeric RNA and viral proteins in different cell-lines.

RESULTS: Immunoblot analysis confirmed coexpression of HCV-E2 and the non-structural protein (NS1) of DENV, but no DENV E protein, suggesting correct processing of the chimeric polyprotein. The detection of chimeric RNA after multiple passages of the supernatant from transfected cells followed by precipitation, RNA-digestion and centrifugation through sucrose suggested release of viral RNA from the transfected (and the infected) cells in membrane protected, particle-like structures. Since inoculated cells showed cytopathogenic alterations, a plaque assay was established. Seven days after addition of the plaque assay medium, cells were stained with Neutral Red. Cells showed clear formation of the typical faint plaques, best visible at a dilution of 1:32 and 1:64. According to the plaque assay, the titer of infectious particles in the supernatant of transfected cells was around 3.5×10^4 pfu/ml, and therefore relatively low.

CONCLUSION: These results suggested autonomous replication of the constructed chimeric HCV/DENV-flavivirus and the formation of a virus like particle with infective properties, which could be used to determine viral binding, attachment and internalization of HCV.

PERMEABILITY STUDIES ON RICKETTSIA RICKETTSII-INFECTED HUMAN CEREBRAL MICROVASCULAR ENDOTHELIAL CELLS

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BACKGROUND: Rickettsioses are arthropod-borne diseases caused by gram-negative obligately intracellular bacteria that belong to the alpha subdivision of the proteobacteria. These diseases are still prevalent in many parts of the world and include Rocky Mountain spotted fever (RMSF, the most common rickettsiosis in the US), epidemic, and endemic typhus caused by *Rickettsia rickettsii*, *R. prowazekii*, and *R. typhi*, respectively. The main target cell is the microvascular endothelium, leading to a disseminated infection whose most severe complications include a vasogenic cerebral edema and non-cardiogenic pulmonary edema. These two complications are responsible for most of the morbidity and mortality seen in humans. *R. rickettsii* and *R. prowazekii* are both Category B select agents and potential bioterrorists due to their high morbidity and mortality in humans and their high infectivity via aerosolization. The mechanisms responsible for increased microvascular permeability are completely unknown.

PURPOSE: To develop an in vitro model to study the kinetics of increased microvascular permeability in human cerebral microvascular endothelial cells and elucidate the underlying molecular mechanisms.

METHODS: Sarcoma-virus transformed human microvascular endothelial cells (SV-HCEC) were used in our experiments. Permeability was evaluated by monitoring the cell monolayers by Electronic Cell Substrate Impedance Sensing (ECIS). Initially, the monolayers were infected with 1-50 MOI of renografin-purified *R. rickettsii* and permeability changes were monitored up to 120 hours after infection in order to study dose-response curves. Subsequent experiments were performed with 15 MOI. Cell death rates were determined by staining monolayers with propidium iodide. The effects of cytokines on endothelial permeability were evaluated by adding TNF- α , IFN- γ , and IL-1 β , 24 hours after infection of the monolayers at concentrations ranging from 0.1 to 1,000 ng/ml. Junctional proteins were studied by immunofluorescence and laser confocal microscopy.

RESULTS: Confluent SV-HCEC monolayers exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in resistance. Permeability increased steadily 2-3 hours after internalization. At 24 hours, increases in permeability ranged from 12% at 1 MOI to 25% at 50 MOI and continued to increase at 48, 72, 96 and 120 hours reaching 50-55% at 120 hours post-infection. Cell death rates were similar in both infected and non-infected monolayers for the first 72 hours at 15 MOI suggesting that changes in permeability during the first 3 days are not due to cell death. Addition of IL-1 β and TNF- α to non-infected monolayers also altered their permeability. At concentrations of 0.1 ng/ml of TNF- α , increases in permeability (which peaked at 5%) were reversible. At 1 ng/ml, the maximum increase was 25% at 50 hours. At 10 ng, a maximum increase of 40% was observed at 72 hours and marked increases in permeability (60-70% at 72 hours when concentrations of TNF- α reached 100 and 1000 ng/ml respectively). Similar changes were observed with addition of IL-1 β . On the other hand, IFN- γ increased the resistance across monolayers at all concentration, with no dose-response relationship. When IL-1 β and TNF- α were added to infected monolayers, a further increase in permeability (8-12% at low concentrations and 20-27% at high concentrations) was observed and was also dose-dependent. Furthermore, at 0.1 ng/ml, the infected monolayer never recovered its baseline resistance, suggesting a synergistic effect between rickettsiae and TNF- α and IL-1 β .

CONCLUSION: Rickettsial infection of microvascular endothelial cell monolayers derived from human brain increases their permeability in a dose-dependent manner. Cytokines such as IL-1 β and TNF- α further increase permeability in such monolayers. IFN- γ seems to "stabilize" the monolayers and to some extent counteract the effects of the other cytokines. Changes in permeability observed during the first three days are not related to cell death.

BACTEROIDES FRAGILIS OMPA: UTILITY AS A LIVE VACCINE VECTOR FOR BIODEFENSE AGENTS: CONSTRUCTION OF AN OMPA DELETANT AND CHARACTERIZATION OF THE FUNCTION OF OMPA

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BACKGROUND: The dangers of bioterror bacterial or viral agents have been of concern to the military and political leaders for decades and have more recently become an issue of public concern as well. Historically, vaccines have been the most efficient method of handling diseases in large populations and live vaccine vectors are particularly appealing. The OmpA bacterial outer membrane protein would be an ideal candidate to serve as the vehicle to carry foreign antigens, such as those from biodefense organisms. The OmpA protein is an outer membrane protein that has four loops exposed on the outer surface of the bacteria. *Bacteroides fragilis*, one of the commensal gut bacteria, is an ideal vehicle for this live vaccine vector. The genetically modified *B. fragilis*, carrying the specific epitopes desired on its modified OmpA, could colonize the gastrointestinal tract and prevent the pathogen or toxin from exiting the GI tract to invade the circulatory system or other organs. Specific peptide epitopes from a variety of potential pathogens, including agents of bioterror, could be engineered into the external loops.

PURPOSE: Our earlier studies characterized the *B. fragilis* OmpA protein and identified the *ompA* gene. The purpose of this study was to construct a *B. fragilis* OmpA deletant and to begin to characterize the function of OmpA, as well as the specific function(s) of the various loops. The OmpA deletant will be used to express various modified forms of the OmpA gene, including specific loop deletants.

METHODS: WAL 108 (*B. fragilis* ADB77, a derivative of *B. fragilis* 638R optimized for use in constructing deletants) was used as the parental strain. WAL 186 (ADB77 Δ ompA) was constructed using a two-step double-crossover technique with the pADB242a suicide vector. Analysis of gene expression was performed by real-time RT-PCR and normalized with measurements of 16S rRNA expression. SDS sensitivity assays were performed by plating WAL 108 and WAL 186 (in log phase growth) on media containing 0.05-0.2% SDS, and incubating for 24-48 hours in an anaerobic jar. Acid and salt sensitivity assays were performed by incubating log phase cells at pH 4.0 for 30 minutes or 5M NaCl for 2 hours, respectively, plating on BHI containing added thymine and incubating for 48 hours in an anaerobic jar. The effect of 200 mM NaCl on cell shape and *ompA* expression was measured after growing WAL 108 and WAL 186 overnight in BHIS broth with or without added 200 mM NaCl. Susceptibility testing was performed using the spiral gradient endpoint method.

RESULTS: We constructed an *ompA*-11 deletant (WAL 186) and confirmed the deletion by sequence analysis of the deletion junction. RT-PCR indicated that all 4 *ompA* ds are transcribed in the parental strain, and confirmed that *ompA*-11 is not transcribed in WAL 186 (the *ompA*-11 deletant). However, we found that *ompA*-14 was also not transcribed in the deletion mutant. No significant change was seen in MICs of a variety of antimicrobials for the deletion mutant compared to the parental strain. WAL 186 was more sensitive than WAL 108 to high salt. Exposure of the parental strain WAL 108 to 5M NaCl for 2 hours resulted in a 3 log₁₀ reduction in growth; WAL 186 did not grow at all after exposure to high salt. Similarly, growth of WAL 108 on media containing 0.05-0.2% SDS resulted in a 3 log₁₀ reduction in growth as compared to growth on media without SDS; WAL 186 did not grow at all on media containing SDS. No change between the WAL 108 and WAL 186 was seen after exposure to low pH. Gram stain analysis showed no change was seen between WAL 108 and WAL 186 grown overnight in normal media (long, somewhat pleomorphic rods), but overnight growth in hypersmolar media (200 mM NaCl) resulted in very small, round forms for both WAL 108 and WAL 186. In addition, expression of *ompA*-11 and *ompA*-14 was diminished in WAL 108 (the parental strain).

CONCLUSION: The expression of *B. fragilis* *ompA*-11 is dependent on the expression of *B. fragilis* *ompA*-11. *B. fragilis* *ompA*-11 is important in maintaining cell structure under stress (e.g., exposure to detergent or high salt). Further, when the cells are exposed to these agents, it responds by shutting down the expression of *ompA*-11 and physically constricting itself to a small, round form (perhaps to lessen the possibility of passage of these agents into the cell). Finally, we have constructed an OmpA deletant for use in determining specific loop functions and designing a vaccine vector.



L-3 RICKETTSIA INFECTION

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Rickettsiae are obligate intracellular alpha-proteobacteria that primarily targets the microvascular endothelium. Two genera are recognized in the family Rickettsiaceae, namely *Rickettsia* and *Orientia*. The genus *Rickettsia* has been further subdivided into spotted fever and typhus groups. In the last two decades, new rickettsial pathogens have been associated with human illness around the world. Clinically, the common denominator in all rickettsioses is the development of a "leaky" microcirculation leading to the most feared complications, namely vasogenic cerebral edema and non-cardiogenic pulmonary edema. Histopathologic studies based on fatal human cases and well characterized animal models show meningoencephalitis and interstitial pneumonitis, amongst others. With the development of powerful research tools, advances in the understanding of rickettsial pathogenesis have been dramatic. Entry into the host cell is followed by rapid escape into the cytoplasm to avoid phagolysosomal fusion, mediated by a newly described phospholipase D. SFG rickettsiae induce actin polymerization via a group of proteins called RickA which promote nucleation of actin monomers via the Arp2/3 complex at one rickettsial pole, propelling the bacteria across the cytoplasm and into neighboring cells. Damage to the host cell is most likely multifactorial. The best well studied mechanism is the generation of reactive oxygen species (ROS) and downregulation of enzymes involved in protection against oxidative injury leading to lipid peroxidation and damage to cellular membranes. The significance of ROS-mediated cellular damage *in vivo* is beginning to be elucidated. Tissue and organ damage is also multifactorial and complex. The main pathogenic mechanism is increased microvascular permeability leading to profound metabolic disturbances in the extravascular compartment. The underlying factors responsible for those changes are beginning to be elucidated *in vitro* and include direct effects of intracellular rickettsiae, cytokines and possibly activated coagulation factors all of which most likely modify interendothelial junctions. Great advances in the understanding of the immune response to both typhus and spotted fever group rickettsiae have also been made. Our knowledge on rickettsial pathogenesis will continue to expand in the near future as new research tools such as *in vivo* and live-cell imaging, multiphoton confocal microscopy, and nanotechnology become more available.

O-59 NITRIC OXIDE (NO) AS A MEDIATOR OF INCREASED MICROVASCULAR PERMEABILITY DURING RICKETTSIAL INFECTION.

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Introduction: Rickettsiae primarily target the microvascular endothelium leading to disseminated infection of the microvasculature. The mechanisms of increased microvascular permeability during rickettsiosis are not well understood. The role of NO as an important anti-rickettsial agent has been well characterized in animal models. We sought to better describe the role NO plays in modulating endothelial tight junctions in microvascular endothelial cells and how this contributes to increased microvascular permeability. **Methods:** Microvascular endothelial cells were grown to confluence on 8W10E gold-coated electrodes and transendothelial electrical resistance was monitored by Electric Cell-substrate Impedance Sensing, or ECIS. The cells were infected with *Rickettsia conorii* (Malish 7) followed by the addition of the NO donors DETA NONOate and SNAP. Similarly treated cells were stained for the tight junction protein occludin and ZO-1 and examined by laser confocal microscopy (LCF).

Additionally, intracellular proliferation of rickettsia in the absence and presence of NO donors was followed by quantitative real-time PCR. **Results:** The addition of NO donors to infected endothelial cells resulted in a marked increase of transendothelial permeability as measured by ECIS. This increase occurred within the first 24 hours of stimulation. Light microscopic observation of endothelial cell morphology revealed no drastic changes in cell shape. However, preliminary staining of the tight junction-associated protein occludin appears to demonstrate disassembly of the tight junction complex by the loss of occludin staining at intercellular borders by LCM. Quantification of intracellular rickettsiae by quantitative real-time PCR is currently in progress. **Conclusion:** The addition of NO donors appears to cause a dramatic and immediate increase in transendothelial permeability in an in vitro model of rickettsial infection. Reorganization of endothelial tight junctions as determined by LCM appears to explain the observed changes in permeability. The ability of these NO oxide donors to limit intracellular proliferation of rickettsiae has not been determined at this point. Future experiments are planned using primary mouse brain microvascular endothelial cells.

O-60 ANKYRIN REPEAT CONTAINING GLYCOPROTEIN (GP200) IN Ehrlichia CHAFFEENSIS AND E. CANIS NUCLEI OF INFECTED CELLS

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Objectives: The objective of this study was to determine the role of gp200 in ehrlichial modulation of host cell membrane and *E. canis* 200 kDa glycoprotein (gp200) and ehrlichial immunoreactive proteins, suggest potential involvement in protein-protein interactions of these glycoproteins in ehrlichial infection. Immunogold electron microscopy was performed on cytoplasmic and nuclear fractions of *E. canis*-infected DH82 cells and from THP-1 cells to confirm the presence of gp200 protein fragments re-expressed and used as probes in immunoprecipitation experiments. Interactions between gp200 and nuclear proteins was localized in the cytoplasm of ehrlichial cells and with the host cell nuclear membrane proteins (Fbp and dsb) were detected. gp200 was detected in the nuclear fractions of infected cells. Two internal gp200 fragments containing ankyrin repeats) also bound to nuclear proteins (65 kDa). **Conclusion:** In this study, we determined that *E. canis* gp200s are translocated to the nucleus and identified two recombinant fragments of gp200 containing ankyrin repeats and identified two recombinant fragments of gp200 containing ankyrin repeats and identified two recombinant fragments of gp200 containing ankyrin repeats and identified two recombinant fragments of gp200 containing ankyrin repeats.

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